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Diagnostic and prognostic role of expressed KRAS and BRAF mutations in MAPK/ERK-driven cancers

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To Dad and Mom

To Linh, Min and Kin

Kính tặng cha mẹ

Thân tặng Linh và các con Min, Kin

ABSTRACT

The aim of this doctoral thesis was to utilize molecular biology techniques to detect expressed KRAS and BRAF mutations in samples at high sensitivity and specificity and evaluate the clinical role of the RNA expression of these mutations in certain MAPK/ERK-driven cancers.

In the early phase of the study, we developed a novel method named extendable blocking probe reverse transcription (ExBP-RT) for detecting expressed mutations at the mRNA level. With this method we were able to detect mutations expressed in mRNA with a very high sensitivity and specificity. The ExBP-RT assay was optimized to detect expressed BRAF and KRAS mutations in a 1000-fold – 6000-fold excess of wild-type mRNA. A further improvement of the method allowed detection of expressed BRAF mutations, in thyroid cancer (TC) tissue, in a 10000-fold excess of wildtype mRNA. This novel strategy not only reveals the presence or absence of low-abundance mutations with an exceptionally high selectivity, but also provides a convenient tool for accurate determination of the mRNA levels of the mutated genes in different settings, such as quantification of allele-specific expression.

We used the ExBP-RT technique to measure the mRNA levels of all seven KRAS mutations at codon 12 and 13 in primary tumour tissue samples of 571 patients with colorectal cancer (CRC). Survival data was analysed to determine the prognostic potential of this novel mRNA-based biomarker. A high level of mutated KRAS mRNA was associated with an inferior 5-year disease-specific survival (DSS). This association was highly significant, but only in left-sided CRC ($P < 0.001$). Thus, the mRNA level of the mutated KRAS allele in primary tumour tissue was found to be highly prognostic in left-sided CRC, but not in right-sided disease.

To study the RNA expression of BRAF mutations as a diagnostic marker in thyroid cancer (TC), we analysed 62 formalin fixed paraffin embedded (FFPE) samples from TC patients. We detected BRAF V600E mutations at the mRNA level in 56,3% (18/32) and on the DNA level in 40,6% (13/32) of thyroid cancer patients, which is in concordance with the reported prevalence of these mutations.

In order to evaluate whether sensitive detection of KRAS and BRAF mutations in mRNA could serve as a biomarker for early detection of malignant transformation in patients at risk of developing colorectal cancer, we analysed esophageal atresia (EA) patient cohorts. The ExBP-RT technique was used to evaluate tissue expression of KRAS and BRAF mutations in endoscopic biopsies from 61 adults, who had been surgically treated for EA in infancy. Despite the presence of histological findings indicating an increased risk of developing cancer, we found no detectable tissue expression of KRAS or BRAF mutations in this cohort.

In conclusion, we successfully established a novel technique – ExBP-RT to detect KRAS and BRAF mutations at the mRNA level with very high sensitivity and selectivity. We found a strong association between the tumour tissue expression of KRAS mutations and prognosis in left-sided, stage III CRC. We also successfully detected and quantified the level of BRAF V600E mRNA in FFPE tissue from thyroid cancer. We could not, however, detect neither KRAS nor BRAF mutations in either of the cohorts of EA patients representing potential pre-malignant conditions.

TIIVISTELMÄ

Väitöskirjatyön tavoitteena oli hyödyntää molekyylibiologisia menetelmiä joilla voidaan havaita ilmentyviä KRAS- ja BRAF-mutaatioita kudosnäytteissä suurella herkkyydellä ja spesifisyydellä, sekä arvioida näiden mutaatioita kantavien RNA molekyylien ilmentymän kliinistä merkitystä tietyissä MAPK/ERK-polkuun liittyvissä syövissä.

Tutkimuksen alkuvaiheessa kehitimme uuden menetelmän mutaatioita kantavien RNA molekyylien havaitsemiseksi lähetti-RNA-tasolla (Extendable Blocking Probe Reverse Transcription: ExBP-RT). Menetelmä optimoitiin havaitsemaan BRAF- ja KRAS-mutaatioita sisältävät lähetti-RNA (mRNA) -molekyylit jopa 10000-kertaisen normaalin genotyypin omaavan lähetti-RNA:n joukosta. Uusi menetelmä paljastaa lähetti-RNA:ssa ilmentyvien mutaatioiden esiintymisen, minkä lisäksi se tarjoaa työkalun mutatoituneiden geenien mRNA-tasojen määrittämiseen eri olosuhteissa.

Käytimme ExBP-RT-tekniikkaa mittaamaan 7 eri KRAS-mutaation mRNA- tasoja 571 paksusuolensyöpäpotilaan kasvainkudosnäytteissä. Uuden mRNA-pohjaisen biomarkkerin ennustepotentiaali arvioitiin analysoimalla eloonjäämistietoja. Korkea mutatoituneen KRAS mRNA:n ilmentymisaste liittyi huonompaan viiden vuoden tautispesifiseen eloonjäämiseen. Korrelaatio oli erittäin merkittävä vasemmanpuoleisessa taudissa ($P < 0,001$) ja mutatoituneen KRAS mRNA-tason todettiin olevan vahva ennustetekijä vasemmanpuoleisessa paksusuolensyövässä, mutta ei oikeanpuoleisessa taudissa.

Seuraavaksi tutkimme BRAF-mutaatioiden RNA-ilmentymää diagnostisena merkkiaineena kilpirauhassyövässä, analysoimalla 62 arkistoitua kilpirauhassyöpäpotilaan kudosnäytettä. Havaitsimme BRAF V600E -mutaatioita mRNA-tasolla 56,3%:lla (18/32) ja DNA-tasolla 40, 6%:lla (13/32) kilpirauhassyöpäpotilaista, mikä vastaa näiden mutaatioiden raportoitua esiintyvyyttä.

Lopuksi selvitimme voisiko KRAS- ja BRAF- mutaatioiden herkkä havaitseminen mRNA-tasolla toimia biomarkkerina pahanlaatuisen muuntumisen varhaisessa havaitsemisessa. Analysoimme ExBP-RT-tekniikkaa käyttäen KRAS- ja BRAF- mutaatioiden ilmentymistä endoskooppisissa biopsiassa 61:llä aikuisella, joita oli lapsena hoidettu kirurgisesti ruokatorviatresian vuoksi. Huolimatta histologisista löydöksistä, jotka viittasivat lisääntyneeseen syövän kehittymisen riskiin, emme löytäneet tästä kohortista KRAS- tai BRAF-mutaatioiden kudosilmentymistä mRNA-tasolla.

Olemme kehittäneet uuden menetelmän - ExBP-RT, KRAS- ja BRAF-mutaatioiden havaitsemiseksi mRNA-tasolla erittäin suurella herkkyydellä ja spesifisyydellä. Menetelmä vaikuttaa lupaavalta ennusteen määrittämisessä paksusuolensyövässä, sekä kilpirauhassyövän varhaisdiagnostiikassa.

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals:

I. Tho Ho, **Kien Dang**, Susanna Lintula, Kristina Hotakainen, Lin Feng, Vesa M. Olkkonen, Emmy W. Verschuren, Tuomas Tenkanen, Caj Haglund, Kaija-Leena Kolho, Ulf-Hakan Stenman, Jakob Stenman. *Extendable blocking probe in reverse transcription for analysis of RNA variants with superior selectivity.* Nucleic Acids Res, 2015. **43**(1): p. e4.

II. **Kien Dang**, Tho Ho, Saara Sistonen, Antti Koivusalo, Mikko Pakarinen, Risto Rintala, Ulf-Hakan Stenman, Arto Orpana, Jakob Stenman. *No tissue expression of KRAS or BRAF mutations in 61 adult patients treated for Esophageal Atresia in early childhood.* Eur J Pediatr Surg. 2018 Oct; **28**(5): 413-419.

III. **Kien Dang***, Tien Tran*, Quynh Huong Pham, Ung Dinh Nguyen, Nhung Thi Trang Trinh, Luong Van Hoang, Son Anh Ho, Ba Van Nguyen, Duc Trong Nguyen, Dung Tuan Trinh, Dung Ngoc Tran, Arto Orpana, Ulf-Håkan Stenman, Jakob Stenman & Tho Huu Ho. *Evaluation of the expression levels of BRAF V600E mRNA in primary tumors of thyroid cancer using an ultrasensitive mutation assay.* BMC Cancer, 2020. **20**(368).

IV. **Kien Dang***, Tho Ho*, Kati Räsänen, Susanna Lintula, Tien V. Tran, Vang Le-Quy, Nhung T.T. Trinh, Harri Mustonen, Arto Orpana, Ulf-Håkan Stenman, Caj Haglund, Jakob Stenman. *Expression levels of mutated KRAS mRNA defines dismal prognosis in left-sided colorectal cancer.* (Unsubmitted).

** These authors contributed equally to the study.*

ABBREVIATIONS

A, T, G, C: adenine, thymine, guanine, cytosine.
AKT: protein kinase B (PKB).
APC: adenomatous polyposis coli.
AREG: amphiregulin.
ARMS-PCR: amplification refractory mutation system-PCR.
AS-PCR: allele-specific PCR.
ASB-PCR: allele-specific PCR with a blocking reagent.
ASK: apoptosis signal-regulating kinase.
BE: Barrett's esophagus.
BRAF: v-Raf murine sarcoma viral oncogene homolog B.
cDNA: complementary deoxyribonucleic acid.
CEA: carcinoembryonic antigen.
CGH: comparative genomic hybridisation.
CI: confidence interval.
CIMP: CpG island methylation pathway.
CIN: chromosomal instability.
CRC: colorectal cancer.
cRNA: complementary ribonucleic acid.
ddPCR: digital droplet PCR.
DEPC: diethylpyrocarbonate .
DNA: deoxyribonucleic acid.
dsDNA: double-stranded template DNA.
DSS: disease-specific survival.
EA: esophageal atresia.
EAC: esophageal adenocarcinoma.
EGFR: epidermal growth factor receptor.
EREG: epiregulin.
ERK: extracellular-signal-regulated kinases.
ExBP: extendable blocking probe.
ExBP-RT: extendable blocking probe - reverse transcription.
FAP: familial adenomatous polyposis.
FFPE: formalin fixed paraffin embedded.
FISH: fluorescence in situ hybridization.
FIT: faecal immunochemical test.

FOBT: faecal occult blood testing.
FPM: functional precision medicine.
GDP: guanosine diphosphate.
GER: gastroesophageal reflux.
GERD: gastroesophageal reflux disease.
GM: gastric metaplasia.
GPCR: G-protein-coupled receptor.
GTP: guanosine triphosphate.
HDI: human development index.
HIV: human immunodeficiency viruses.
HR: hazard ratio.
IBD: inflammatory bowel disease.
IM: intestinal metaplasia.
IQR: interquartile range.
JAK: Janus kinase.
JNK: Jun amino-terminal kinases.
KRAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog.
L-DNA: long-form DNA.
LOH: loss-of-heterozygosity.
LS: Lynch syndrome.
MAP: mitogen-activated protein.
MAPK, MK: mitogen-activated protein kinase.
MAPKK, MAP2K, MEK, MKK: mitogen-activated protein kinase kinase.
MAPKKK, MAP3K, MEKK: mitogen-activated protein kinase kinase kinase.
MLH1: mutL homolog 1.
MLK: mixed lineage kinase.
MMR: mismatch repair.
mRNA: messenger ribonucleic acid.
MSI: microsatellite instability.
MSK: mitogen- and stress-activated kinases.
mt: mutant.
NGS: next-generation sequencing.
NRAS: neuroblastoma RAS.
OS: overall survival.
PASA PCR: amplification of Specific Alleles.
PCR: polymerase chain reaction.
PI3K: phosphoinositide 3-kinases.

PIK3CA: phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha.

PJS: Peutz-Jeghers syndrome.

PM: personalized medicine.

PRAK: p38-regulated/activated protein kinase.

PTC: papillary thyroid carcinoma.

qPCR: quantitative PCR.

qRT-PCR: quantitative RT-PCR.

RAS, Ras: rat sarcoma viral oncogene homolog.

RNA: ribonucleic acid.

RNA-seq: RNA sequencing.

RT: reverse transcription.

RT-PCR: reverse transcription - polymerase chain reaction.

RTK: receptor tyrosine kinases.

SAPK: stress-activated protein kinases.

scRNA-seq: single cell RNA sequencing.

SD: standard deviation.

SNP: single nucleotide polymorphisms.

SPS: serrated polyposis syndrome.

ssDNA: single stranded DNA.

T-x-Y: threonine-x-tyrosine.

TAK: TGF-β-activated protein kinase.

TAO: thousand-and-one amino acids.

TC: thyroid cancer.

TEF: tracheoesophageal fistula.

TEY: threonine-glutamate-tyrosine.

TGF: transforming growth factor.

TGY: threonine-glycine-tyrosine.

Tm: melting temperature.

TP53: phosphoprotein p53, tumor suppressor p53.

Tpl2: tumor progression locus 2.

TPY: threonine-proline-tyrosine.

UC: ulcerative colitis.

WGS: whole genome sequencing.

wt: wildtype.

INTRODUCTION

Cancer is the most common cause of death in the industrialized world. The population affected on a global level is rapidly increasing, due to ageing of the population in many developing countries. Unfortunately, many cancers are diagnosed at a late stage when the disease has already progressed locally or by metastasis. When distant metastasis has occurred, the prognosis is usually poor, and curative treatment is rarely possible [1]. Diagnosis at an early stage and accurate prognosis is crucial for timely and relevant treatment to save patient lives. Carcinogenesis involves a cascade of genetic aberrations and genetic changes, which can be a very early sign of a cancer [2]. Genetic biomarkers are discovered at an accelerating pace, and they are essential for understanding the pathways of cancer development and finding effective treatment strategies. Genetic biomarkers are also rapidly emerging as an integral part of cancer therapy, not only for early diagnosis, but also for individualized treatment through the development of functional precision medicine (FPM) strategies [3, 4].

Currently there are many technologies for examining genetic changes in cancer, from the chromosomal level to the DNA sequence, as well as epigenetic changes such as DNA methylation patterns and variations in RNA or protein expression. The RAS-RAF-MEK-ERK-MAP kinase pathway plays an important role in the development of many cancers, and KRAS mutations are among the most commonly occurring genetic changes in cancer. The occurrence of KRAS mutations in the DNA of tumour tissue has been established as predictive of response to anti-EGFR therapy in colorectal cancer and sensitive detection of KRAS mutations is an appealing strategy for early detection of many pre-malignant conditions. The prognostic significance of KRAS mutations in tumour DNA is, however, still under debate.

Early detection of mutations in tumour tissue, at a high level of sensitivity, is challenging due to the overwhelming amount of wildtype DNA in surrounding normal tissues as well as in blood cells and in the tumour tissue itself. Genetic testing, so far, has focused on detecting mutations in DNA and there is limited information on the expression level of the mutated alleles, and the possible prognostic relevance of mutated mRNA in tumour tissue. In this doctoral thesis project, we have developed and applied a novel technique for measuring the level of expressed mutations in tumour tissue and evaluated the diagnostic and prognostic potential of expressed KRAS and BRAF mutations in different clinical settings.

REVIEW OF THE LITERATURE

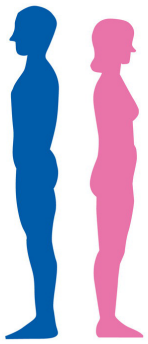
1. THE ROLE OF DIAGNOSIS AND PROGNOSIS IN MAPK/ERK-DRIVEN CANCERS

1.1. Colorectal cancer

Overview

Colorectal cancer (CRC), arising in the large intestine (colon or rectum), is the most common gastrointestinal cancer. Globally, CRC is the third most common cancer in men and the second most common in women (2018) [5]. Overall, CRC is the second most common cancer and the third most common cause of cancer death [5-7].

Estimated New Cases

	Estimated	Portion	Males	Females		Estimated	Portion
Prostate	191,930	21%			Breast	276,480	30%
Lung & bronchus	116,300	13%			Lung & bronchus	112,520	12%
Colon & rectum	78,300	9%			Colon & rectum	69,650	8%
Urinary bladder	62,100	7%			Uterine corpus	65,620	7%
Melanoma of the skin	60,190	7%			Thyroid	40,170	4%
Kidney & renal pelvis	45,520	5%			Melanoma of the skin	40,160	4%
Non-Hodgkin lymphoma	42,380	5%			Non-Hodgkin lymphoma	34,860	4%
Oral cavity & pharynx	38,380	4%			Kidney & renal pelvis	28,230	3%
Leukemia	35,470	4%			Pancreas	27,200	3%
Pancreas	30,400	3%			Leukemia	25,060	3%
All Sites	**893,660**	**100%**			**All Sites**	**912,930**	**100%**

Estimated Deaths

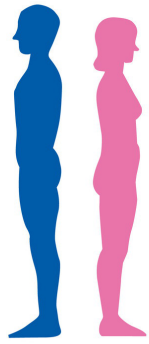
	Estimated	Portion	Males	Females		Estimated	Portion
Lung & bronchus	72,500	23%			Lung & bronchus	63,220	22%
Prostate	33,330	10%			Breast	42,170	15%
Colon & rectum	28,630	9%			Colon & rectum	24,570	9%
Pancreas	24,640	8%			Pancreas	22,410	8%
Liver & intrahepatic bile duct	20,020	6%			Ovary	13,940	5%
Leukemia	13,420	4%			Uterine corpus	12,590	4%
Esophagus	13,100	4%			Liver & intrahepatic bile duct	10,140	4%
Urinary bladder	13,050	4%			Leukemia	9,680	3%
Non-Hodgkin lymphoma	11,460	4%			Non-Hodgkin lymphoma	8,480	3%
Brain & other nervous system	10,190	3%			Brain & other nervous system	7,830	3%
All Sites	**321,160**	**100%**			**All Sites**	**285,360**	**100%**

Figure 1: Ten Leading Cancer Types for the Estimated New Cancer Cases and Deaths by Sex, United States, 2020 (Cancer statistics, Siegel, R., 2020) [8]. Reuse permission granted by Rebecca Siegel and American Cancer Society.

European countries have the highest incidence and mortality related to CRC [9]. A high incidence of CRC is also reported in North America, and Oceania, whereas the incidence is lowest in south and central Asia and Africa [10, 11]. The estimated incidence rates of colorectal cancer in countries

with higher Human Development Index (HDI) are about 5 times higher than in countries with a lower HDI. In Australia and Europe, the rates are 35–42/100 000 in men and 24–32/100 000 in women, compared to 7/100 000 in men and 6/100 000 in women in West Africa, and 6/100 000 in men and 4/100 000 in women in South Asia [12]. The incidence of CRC is currently increasing rapidly in many countries with a previously low incidence, such as Spain, Eastern Europe and East Asia, a phenomenon that has been ascribed to changes in dietary patterns towards a Western lifestyle [11, 13].

Risk factors

The risk factors of CRC can be divided into two groups of unmodifiable factors (hereditary CRC) and modifiable factors (sporadic form). The unmodifiable factors include age, gender, ethnicity, family history of CRC, genetic predisposition syndrome such as familial adenomatous polyposis (FAP), Lynch syndrome (LS), MUTYH-associated polyposis, Peutz-Jeghers syndrome (PJS) and serrated polyposis syndrome (SPS) [14, 15]. LS is the most common the genetic predisposition syndromes of colorectal cancer (CRC) with an incidence of 3–5% of all CRC, followed by FAP, which accounts for approximately 1% of the all CRC cases [16]. The prevalence of FAP is about 1/10000 – 30000 in both men and women [15]. If there's no early diagnosis and treatment, almost all FAP patients develop CRC by the age of 40, whereas colon cancer usually occurs after 10 years of polyp onset [15]. The modifiable factors include inflammatory bowel disease (IBD), lifestyle factors such as lack of physical activity, diet with low in fruit and vegetables, overweight and obesity, alcohol consumption and smoking [11, 14]. Among these, age over 50 years conveys the highest risk for developing CRC.

There're many risk factors which contribute to the development of CRC, but the actual cause is still unknown. In most cases of CRC, no single risk factor can be pointed out and about 95% cases of CRC are considered sporadic. Only 5% cases arise in individuals with inherited unmodifiable risk factors, when gene mutations, or changes, are passed within a family from 1 generation to the next [17].

Molecular pathogenesis

The molecular mechanism of CRC development is important in the clinical management of the disease, because it determines the diagnosis, the prognosis and the response to treatment [11]. Hereditary syndromes contribute to about 3–5% of all CRC and they are high valuable models for studying the molecular pathogenesis of CRC [11]. The two most common types of hereditary CRC are FAP and Lynch syndrome. FAP is autosomal dominant disorder and caused by a germline mutation in the adenomatous polyposis coli (APC) gene. FAP-associated cancers usually develop from the classic adenoma–carcinoma sequence, whereas LS-associated cancers develop via microsatellite instability resulting a deficient mismatch repair [15]. The disease risk increases due to the inherited inactive gene allele. The probability of losing the only functional gene allele is much higher than randomly losing two functional alleles in a cell.

Understanding the development of sporadic CRC is more challenging due to the complex and heterogeneous nature of the disease. Sporadic conventional adenomas have been found to be the most common premalignant precursor lesions and contributed about 65% of CRCs [11], following

by serrated precursor lesions (30%), hereditary syndromes (3-5%) and IBD (1%) [18, 19]. Several genetic and epigenetic events are considered to be involved in a multistep tumorigenesis, leading to the development of CRC [6]. The total number of accumulated genetic mutations is more important than their order, and APC mutations are known as the initiating event with multistep genetic model [15, 20].

Three major molecular pathways leading to CRC have been described, including chromosomal instability (CIN), microsatellite instability (MSI), and the CpG island methylation pathway (CIMP) [15]. These pathways are not necessary mutually exclusive but can occur simultaneously. CIN is the most common pathway of CRC development and contributes of about 70% of sporadic cases. CIN is characterized by the accumulation of structural chromosomal abnormalities, mostly by chromosomal rearrangements and loss-of-heterozygosity (LOH) at tumour suppressor gene loci. In addition, CIN cases usually come with accumulation of chromosomal aberrations affecting several oncogenes and tumour suppressors, such as APC, KRAS, PIK3CA, BRAF, SMAD and TP53. The MSI pathway is a contributing factor in 15% of the sporadic CRC cases. Microsatellites are regions harbouring repeat sequences of 1-6 nucleotide base pairs. Microsatellite instability causes a decreased binding affinity of DNA polymerases, resulting in accumulation of multiple mutations. In MSI, DNA mismatch repair (MMR) is unable to function normally, leading to the accumulations of mutations in the microsatellite regions, including insertions, deletions, and nucleotide substitutions. The inactivation of MMR genes seems to accelerate, rather than initiate, CRC development [11]. The CIMP pathway is characterized by widespread CpG island methylation, that can be found in most sporadic cases of MSI-positive CRC, with tumours usually located in the right colon. Interestingly, BRAF mutations exclusively occur in CIMP positive CRC. Therefore, CIMP positive tumours can be divided in two types: CIMP-high related to BRAF mutations, MLH1 methylation; and CIMP-low related to KRAS mutations [21].

Diagnosis and prognosis

Classification of CRC is crucially important for determination of the prognosis and selecting the optimal treatment protocol for each patient. Initial diagnosis of colorectal cancer is usually made histologically from biopsy samples taken during a diagnostic endoscopy [11]. Staging, on the other hand, is based on histological examination of the surgical resection specimen containing both tumour and lymph nodes, as well as on radiological determination of the presence or absence of metastases. Staging in CRC is based on the TNM8 staging system, including local invasion depth (T), regional lymph node involvement (N) and distant metastases (M) [22].

Patients with localized tumours, without systemic disease are treated with surgery, followed by adjuvant chemotherapy in selected cases. Preoperative radiotherapy or chemoradiotherapy is given in rectal cancer in order to reduce the tumour volume and improve the resectability. Adjuvant systemic chemotherapy is recommended for high-risk stage II and stage III CRC patents with poor prognostic features, such as a perineural, vascular invasion, or high-grade histology [22]. The serum marker carcinoembryonic antigen (CEA) is elevated in most patients, and it is widely used for monitoring of treatment and post-treatment surveillance.

Survival in CRC is mainly dependent on stage. The 5-year survival rates for local stages I and II disease are 93.2% and 82.5%. Corresponding figures in locally advanced stage III disease and primarily metastasized stage III disease are 59.5% and 8.1% respectively. In stage IV, primarily metastasized disease, survival is poor despite aggressive treatment with all currently available treatment modalities [23]. Approximately 20-25% of newly diagnosed CRC patients present with metastatic disease and 30-50% develop metastasis after treatment, contributing to a high mortality rate [24]. The 5-year survival rate of patients with metastatic CRC is only 11% [6]. In addition, there is a well-established difference in prognosis between right sided and left sided CRC [23]. Early diagnosis is crucially important for the successful treatment of CRC. Early detection of pre-malignant lesions or localized CRC is not only critical for the survival of the individual patient, but also for improving the survival rate of CRC in general [25]. Screening of high-risk patients can allow for early diagnosis, curative treatment, and an increased chance of survival.

New diagnostic and prognostic biomarkers now are emerging as an urgent key for avoiding CRC-related deaths [26]. The interconnections between molecular pathogenesis, prognosis, and response to therapy has become apparent during the past two decades [11]. Molecular characterization of the tumour is increasingly important for the identification of specific prognostic subgroups and sensitive molecular detection techniques are being utilized for early identification of predisposing conditions. For the rapidly developing concepts of personalized medicine (PM) and functional precision medicine (FPM), molecular characterization of the tumour is centrally important to allow transition from conventional cytotoxic drugs to molecular biomarker-driven selection of the most suitable agents [24]. Currently, many studies are focusing on molecular testing to guide targeted treatment for CRC patients. Targeted adjuvant therapy with anti-epidermal growth factor receptor antibodies, is the most widely used treatment regimen and successful treatment is dependent on the absence of KRAS mutations. The mutations in KRAS exons 3, 4 or NRAS exons 2, 3, 4 can predict a lack of benefit from anti-EGFR antibodies, but their effect on the efficacy of anti-EGFR treatment is still under investigation [27]. In addition, CRC patients can also benefit from testing for microsatellite instability and the loss of heterozygosity in chromosome 18q, for guiding therapeutic decisions of the administration of 5-fluorouracil.

CRC is a multifactorial disease, with a strong hereditary component in 6% of cases. In sporadic cases, certain genetic mutations such as the BRAF V600E, cause some tumours to be more aggressive. RAS mutations are known to confer resistance to EGFR inhibitor therapy and genetic testing for RAS mutations is considered mandatory prior to initiation of second line treatment in recurrent CRC. Personalized medicine implies individual tailoring of the medical treatment for each patient based on predisposing factors, such as family history of inherited diseases and conditions, as well as on genomic profiling, including both somatic mutations and genetic variants, as well as mutations and other aberrations found in the tumour. Molecular characterization of the tumour allows identification of specific targets for treatment that cannot be identified by traditional techniques, such as tumour histology or immunohistochemistry. Potential benefits of PM are improving clinical outcomes by targeting treatment at specific cellular functions and decreasing treatment-related toxicity by avoiding conventional cytotoxic therapy when it is unlikely to benefit the patient. Furthermore, economic benefits include limiting the prescription and reimbursement

of drugs to patients whom most likely to benefit. [24]. Risk-stratification of patients based on molecular tumour characteristics represents an important strategy for increasing the effect of treatment and ultimately improving survival in CRC [28].

Surveillance and screening

The core components of comprehensive cancer control are prevention, screening, early diagnosis, treatment, palliative and survivorship care [29]. Prevention is the most cost-effective strategy from a public health perspective. Due to the multifactorial evolution of CRC prevention alone is, however, not enough and globally, millions of people will still develop CRC despite prevention efforts. The most common current screening techniques for CRC are faecal occult blood testing (FOBT), flexible sigmoidoscopy or colonoscopy and faecal immunochemical test (FIT). Colonoscopy is regarded as a gold-standard examination to out rule CRC in a patient at risk, due to the ability to examine the whole colon and biopsy or remove any identified lesions for pathological examination. Colonoscopy with biopsies is the most sensitive and specific of all CRC screening methods (80-95% of sensitivity and 95-100% of specificity). An added advantage is that curative polyp removal is possible during the procedure. Examinations should start at the age of 50 years, and be repeated every 10 years, unless otherwise indicated owing to higher risk or other criteria [30]. The application of colonoscopy as a screening technique is, however, limited by its invasive nature and high cost [31, 32]. FOBT screening, which is a non-invasive and substantially more affordable screening technique has been shown to reduce CRC mortality by 16 % compared to a reduction of 30% that has been achieved with flexible sigmoidoscopy. The lower sensitivity of FOBT is particularly attributed to the detection of colonic polyps. Another limitation of FOBT screening is a relatively low specificity with several potential sources of a false positive screening result [33]. There is an urgent need for development of novel diagnostic tools with high sensitivity and specificity for detection of pre-malignant or early-stage malignant lesions to allow cost-effective large-scale screening of CRC. Recent advances in genomics, such as DNA microarray and massive parallel sequencing techniques, as well as proteomic methods such as mass spectrometry, provide efficient tools for the discovery of novel biomarkers [34-37]. Recently identified potential non-invasive screening techniques for CRC include nucleic acid biomarkers such as DNA mutations, long-form DNA (L-DNA), microsatellite instability, epigenetic biomarkers such as DNA methylation patterns and RNA expression profiles as well as protein biomarkers in cancer cells that are released into serum or stool.

There is a consensus that persons with certain warning signs are at an increased risk of developing CRC and should be under surveillance. Patients with a family history of colorectal cancer (a first-degree relative with early-onset CRC or multiple first-degree relatives with CRC) should be screened with colonoscopy more frequently and starting at a younger age. Other warning signs include a personal or family history of FAP or Lynch syndrome, a personal history of colorectal polyps or inflammatory bowel disease (IBD), such as Crohn's disease or ulcerative colitis (UC). Screening and surveillance for the different high-risk groups can be generally divided into two categories: familial colorectal cancer syndromes and IBD [30].

1.2. Esophageal atresia

Overview

Esophageal atresia (EA) is a congenital malformation, in which the esophagus ends in a blind-ended pouch rather than connecting to the stomach. A tracheoesophageal fistula (TEF) between the blind ending pouches of the esophagus is present in most cases. EA occurs with frequency of 1 in 2,500 to 4,500 live births [38-41] and with a prevalence of 2.66 per 10,000 pregnancies in Europe [42].

Malignant transformation

Patients with EA have a high survival rate of 93-95% [43], but despite surgical correction in the new-born period, most patients will experience gastroesophageal reflux (GER) later on with a reported incidence of up to 67% [44]. Chronic GER can lead to esophagitis, anastomotic strictures, and metaplastic epithelial changes like gastric metaplasia (GM) or intestinal metaplasia (IM). This condition is called Barrett's esophagus (BE). Adult patients born with esophageal atresia have high incidence rates of gastro-esophageal reflux symptoms as well as histological signs of esophagitis and Barrett's esophagus [45-47]. Barrett's esophagus is known as a pre-neoplastic condition and a risk factor of esophageal adenocarcinoma (EAC), that presents a high mortality rate [48-50]. Although neoadjuvant chemoradiation therapy followed by surgery can be effective in treatment for EAC, about 60% of patients do not respond to neoadjuvant chemoradiation, reducing chances of successful surgery [51, 52].

Intestinal metaplasia (IM) also constitutes a risk factor for developing EAC and adenocarcinoma occurs in 0.1-2.9% of patients with IM [53]. Intestinal metaplasia has been described in 5-11% of adults and under 3% of adolescents treated for EA in infancy, a 4-fold higher incidence than in the general population [45, 54-56]. There is no information on the prevalence of IM in younger children treated for EA [44]. In the general paediatric population, the prevalence of IM has been shown to be 0.12% among patients without signs of GER disease (GERD) [57]. Due to the increased risk of EAC development in patients treated for EA, early and lifelong surveillance programs including endoscopic biopsies have been suggested for early detection of any malignant transformation [58].

Surveillance and screening

Currently, surveillance for early signs of EAC in EA patients is being applied in many countries [59]. EA patients are recommended to undergo annual screening with endoscopic biopsies taken if any abnormal epithelial changes are found. Screening of BE-associated adenocarcinoma by endoscopy in the general population is a worldwide clinical practice. Random endoscopic biopsies are recommended to be taken in all 4 quadrants and each 2 cm of columnar epithelium [59]. Other screening techniques that have been proposed, but not universally applied, include Lugol chromoendoscopy, cytology techniques, serum markers detected by immunoassay techniques or micro-RNAs [60]. Studies examining KRAS and BRAF mutations in BE and EAC tissues have utilized DNA-based detection techniques such as sequencing or selective polymerase chain reaction [61, 62]. Very few studies looking at blood biomarkers have been performed, but most suggest that these should be used in the future in combination with other screening techniques to

optimize the results [63]. Micro RNAs MiR-330-5p, miR-221 have been evaluated for this purpose [64].

1.3. Thyroid cancer

Thyroid cancer (TC) is the most common endocrine cancer and it accounts for 1–2% of all cancers [65]. Papillary thyroid carcinoma (PTC) is the most common malignant thyroid neoplasm, constituting 80–90% of all thyroid malignancies [66]. Genetic changes occur early in the development of PTC, and BRAF and KRAS mutations are frequently found [67, 68]. Mutations in BRAF occur in 29–69% of PTC, making it is the most common molecular variants [69, 70]. In addition, studies have shown that BRAF V600E mutations might be associated with an aggressive phenotype [71], higher rates of disease recurrence and a shorter disease-free and overall survival [72]. Many studies have concluded that BRAF mutations cannot be considered as a marker of a poor-outcome, but it could be valuable as a diagnostic marker and for post-treatment surveillance. [73, 74]. Among other molecular mutations, RAS mutations can promote thyroid malignancies through the MAPK-ERK or PI3K/AKT pathways [75]. Mutations in RAS has been found in 0–10% of PTCs [70].

2. THE ROLE OF KRAS, BRAF MUTATIONS IN CANCER

2.1. The MAP kinase pathway

The MAP kinase pathway (also known as the MAPK/ERK pathway or the Ras-Raf-MEK-ERK pathway) is one of the most important pathways in cancer development. Through a chain of activation of extra- and intra-cellular proteins, the MAP kinase pathway communicates a signal from the cell surface to the nucleus, that regulates cell proliferation, differentiation and death [76]. Mitogen-activated protein kinases (MAPK) include several protein kinases that share similar substrate recognition sites and confer signalling through a two-step phosphorylation event. The key components of the pathway are divided into MAPK, MAPK kinase (MAPKK / MAP2K) and MAPK kinase (MAPKKK / MAP3K). The MAPKKK directly phosphorylates and activates the MAPKK, then activates the MAPK. When the MAPK is activated, it phosphorylates substrates of the cytosol and nucleus, which makes changes of protein function and gene expression. As result, the appropriate biological responses are then executed (**Figure 2**).

MAP kinases are divided into three main families: ERKs (extracellular-signal-regulated kinases), JNKs (Jun amino-terminal kinases), and p38/SAPKs (stress-activated protein kinases). The difference of these families comes from the T-x-Y (threonine-x-tyrosine) motif of activation segment, as well as the regulation agents and biological responses.

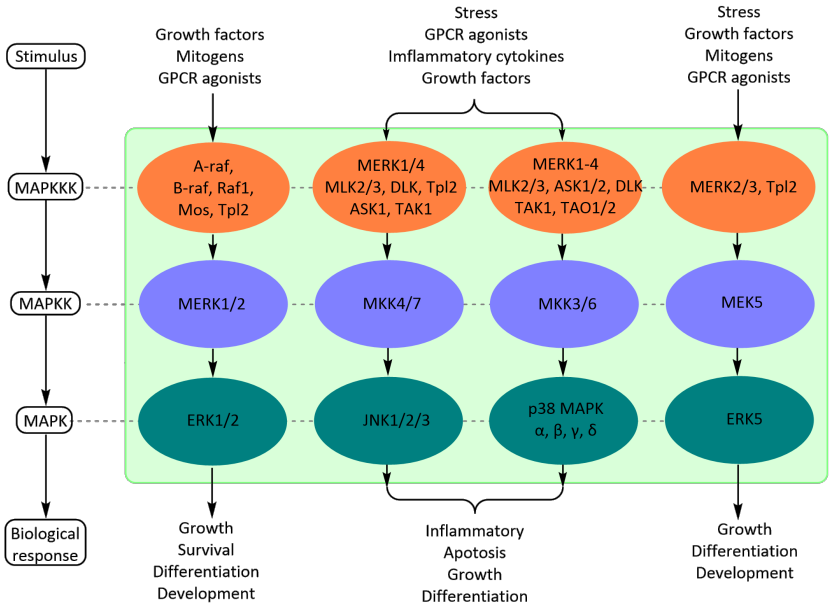


Figure 2: MAPK pathway (Sketch using ChemDraw Professional v20.0, licensed by University of Helsinki)

The ERK family contains a TEY (threonine-glutamate-tyrosine) motif in the activation segment. They can be divided into two groups: the classic ERKs (ERK1 and ERK2) and the larger ERKs (such as ERK5). The classic ERK1/2 group responds mainly to growth factors and mitogens, inducing cell growth and differentiation. Important upstream regulators of classic ERK1/2 group include cell surface receptors, receptor tyrosine kinases (RTK), G-protein-coupled receptors (GPCR), and the small GTPases Ras, Rap. MAPKKs of the classic ERK1/2 are MEK1, MEK2, and the MAPKKKs of the classic ERK1/2 are Mos, Tpl2, which are members of the Raf family. The MAPK-ERK pathway is the best described module of MAPK pathway. It is involved in about one-third of all human cancers and has an important role in cancer development [77].

The JNK family contains a TPY (threonine-proline-tyrosine) motif in the activation segment (JNK1, JNK2, and JNK3). The JNK module is activated by environmental stresses, such as ionizing radiation, heat, oxidative stress, DNA damage, inflammatory cytokines, and growth factors. The JNK module plays an important role in apoptosis, inflammation, cytokine production, and metabolism. MAPKKs of the JNK module are MKK4, MKK7, and the MAPKKKs of the JNK module are MEKK1, MEKK4, MLK2, MLK3, ASK1, TAK1, and Tpl2.

The p38 family contains a TGY (threonine-glycine-tyrosine) motif in the activation segment (p38α, p38β, p38γ, and p38δ). The p38 module is activated by environmental stress, inflammatory cytokines. It contributes mainly to inflammation, apoptosis, cell differentiation, and cell cycle regulation. Some important substrates of p38 family are the downstream kinases MK2/3, PRAK, MSK1, MSK2, and various transcription factors. The MAPKKs of the p38 module are MKK3, MKK6, and the MAPKKKs of the p38 module are MLK2, MLK3, MEKKs, ASKs, TAK1, TAO1 and TAO2.

2.2. KRAS mutations

The KRAS protein is an element of the MAPKKK module, a part of the MAPK-ERK pathway. It is downstream of extracellular signalling and upstream of MAPKK (MEK1/2). The KRAS protein is small G protein (GTPase), which converts GTP into GDP. In this way, it acts like a switch that is turned on (activated) when binding to GTP and turned off (inactivated) when converting GTP to GDP. When the KRAS protein binds to GDP, it will not transmit signals to the cell nucleus. The KRAS protein contributes to important processes in the nucleus, such as proliferation, differentiation, apoptosis, cell adhesion, and cell migration. The KRAS gene which encodes the KRAS protein, is a proto-oncogene. Normally it regulates the propagation of the cell. When it is mutated, it can become an oncogene and potentially cause cancer. Mutations of the KRAS gene can change the structure and function of the KRAS protein. KRAS mutations are found in 30% of all human tumours [78-80] including lung cancer, pancreatic cancer, colorectal cancer, ovarian cancer, as well as prostate and gastric cancers [81]. KRAS mutation have been found to confer resistance to anti-EGFR treatment in colorectal cancer, which is based on blocking the EGFR receptor with a monoclonal antibody, such as cetuximab. Companion diagnostics for KRAS mutation status is considered mandatory before initiating treatment with EGFR inhibitors [82]. Approximately, 85-90% of KRAS mutations appear in codon 12 and 13. Mutations of KRAS codons 61 and 146 occur less frequently and each represent about 5% of all KRAS mutations [82-86].

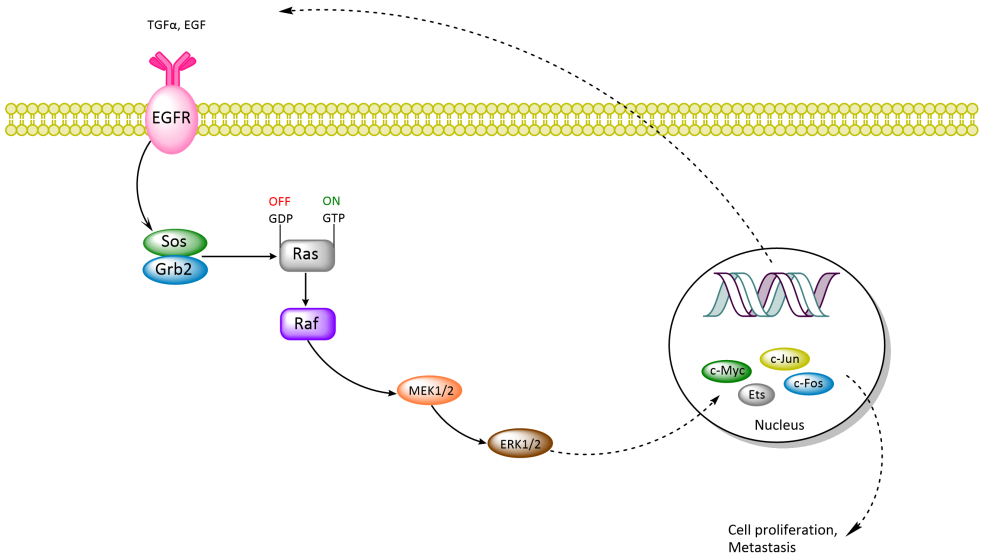


Figure 3: MAPK-ERK pathway (Sketch using ChemDraw Professional v20.0, licensed by University of Helsinki)

Although the association between tumour KRAS mutations and resistance to treatment with anti-EGFR monoclonal antibodies has been firmly established, the prognostic relevance of KRAS mutations remains controversial [87-91]. On the other hand, the expression of Ras p21 protein has been reported to be a prognostic indicator in patients with rectal cancer [92], and to correlate with

the malignant potential of pre-cancerous lesions and malignant tumours in the colon and rectum [93]. Accordingly, the phenotypes of affected cells or tissues in CRC could be affected by the expression level of the mutated KRAS allele [94]. The prognostic value of KRAS mutations has been evaluated in several studies but a correlation between KRAS mutations and a poor prognosis has been established only in metastatic CRC [82]. KRAS mutation testing in tumour tissue has been proposed as a prognostic and predictive biomarker in this group of patients [95].

2.3. BRAF mutations

Like KRAS, the BRAF protein is a part of the MAPKKK module of the MAP-ERK pathway. The BRAF protein is an effector of KRAS. It becomes active when bound to KRAS-GTP. The active BRAF kinase phosphorylates and activates the MEK1/2 downstream cascade with subsequent activation of MAPK (ERK1/2) and transmittal of signals to the nucleus. The BRAF protein plays an important role in cell division, differentiation, and secretion [82]. The BRAF V600E mutation is present in about 8-10% of CRC cases. Many studies have reported that BRAF may act as predictive or prognostic indicator in patients with metastatic CRC that have been treated with cetuximab. Metastatic CRC patients with BRAF mutations in the tumour tissue, have a shorter survival versus patients with wildtype BRAF in the tumour tissue. There is a known association between BRAF mutations and MSI in CRC and both markers are in clinical use for evaluation of tumour aggressiveness. BRAF mutations are associated with a poor prognosis especially in patients with a right-side tumour. [82].

3. CURRENT MUTATION DETECTION TECHNIQUES

3.1. PCR-based methods

The polymerase chain reaction (PCR) is one of the most widely used techniques in molecular biology. PCR is based on the exponential amplification of nucleic acids using a thermostable DNA polymerase [96]. PCR is a robust, powerful tool for detection and quantification of nucleic acids, with high sensitivity and specificity. PCR has revolutionized the field of nucleic acid analysis and it has been widely integrated in clinical diagnostics [96].

In PCR, two synthetic oligonucleotides primers are used to anneal to opposite strands of the double-stranded template DNA target flanking the region of interest. The PCR process consists of three steps: denaturation, primer annealing to the single stranded DNA (ssDNA) strands, and primer extension. By repeating these steps for a number of times, usually 30 to 40 cycles, the resulting DNA target sequence will be amplified exponentially, resulting in billions of copies of the PCR product, the so-called "amplicon." The PCR reaction reaches a plateau phase after a number of cycles, when exponential amplification ends due to several different reasons related to consumption of reagents and accumulation of amplification products in the reaction. Amplicon detection can be performed in real-time during the exponential phase of the reaction, or as end point detection, at the plateau phase. Widely used techniques for end-point detection include agarose gel electrophoresis with ethidium bromide or SYBR Green staining, polyacrylamide gel electrophoresis with fluorescent or radioactive labelling, as well as Southern blotting or phosphor-

imaging. Disadvantages of classical end-point detection techniques are the use of hazardous chemicals and the risk of laboratory contamination. Real time detection technologies largely circumvent these problems.

Reverse transcription - Polymerase chain reaction (RT-PCR)

RT-PCR is a technique in which RNA is used as a starting material for PCR amplification. RNA is converted to complementary DNA (cDNA) by reverse transcription (RT), and the resulting cDNA is amplified by PCR [97]. RT-PCR is known as the most sensitive technique for mRNA detection. It is primarily used for qualitative demonstration of the presence of specific RNAs. In addition to qualitative RNA detection, RT-PCR can be used as a pre-amplification technique prior to cloning or sequencing. Quantitative RT-PCR (qRT-PCR) is usually performed for quantitative analysis of the level of certain RNAs of interest in a sample.

As a research tool RT-PCR can be used for studying the genomes of viruses whose genomes are composed of RNA, such as influenza viruses, retroviruses, and the corona viruses. RT-PCR also enables to examination of expressed variant transcripts of any specific gene. In diagnostic laboratories testing for various alternative splicing variants or fusion transcripts are coming into clinical use. Often a large genomic structural variation is best detected at the mRNA level. There are several ongoing initiatives that utilize RT-PCR for sensitive detection of tumour cells based on unique mRNA transcripts. Since mRNA transcripts often are present in significantly greater copy numbers than DNA in a cell, RT-PCR has the potential to be utilized for sensitive detection of circulating or occult tumour cells in tissues. Thus RT-PCR has the potential for analysis of biomarkers to aid clinical cancer diagnostics and monitoring response to therapy [98].

Multiplex-PCR

Multiplex-PCR is used to amplify several different DNA sequences simultaneously in single PCR reaction tube. Multiple primers are typically included in a single PCR mixture to produce multiple amplicons that are specifically detected in real-time or using end-point detection techniques. The advantages of this technique are saving time and reagents by acquiring information on multiple target genes or RNAs at a time, in a single test-run. Technical challenges of multiplex PCR include the optimization of PCR primer design so that all primer pairs can function at the same annealing temperatures without primer dimer formation during PCR [97]. Multiplex-PCR has been used for analysis of microsatellites and single nucleotide polymorphisms (SNP) and other mutations, as well as for simultaneous detection of multiple infectious agents in the same sample.

AS-PCR

Allele-specific PCR (AS-PCR) is a technique used for detection of point mutations or SNPs in human DNA [99]. Variations of AS-PCR include ARMS-PCR (Amplification Refractory Mutation System-PCR) or PASA (PCR Amplification of Specific Alleles). AS-PCR relies on a mismatch in the 3' end of the PCR primer to achieve selective amplification of alleles mutated at single nucleotide positions. AS-PCR primers are designed to have a nucleotide at 3'-end which is

complementary to specific point mutation. Taq polymerase is typically used for amplification because of its absence of 3' to 5' exonuclease proofreading activity. High-fidelity DNA polymerases, that have this activity, cannot be used in AS-PCR.

AS-PCR has previously been widely used in clinical diagnostics of genetic and infectious diseases [100]. It's an accurate method for detection of SNPs in single-gene disorders and it has for long been regarded as the gold standard method for inherited disorders like sickle cell anaemia and thalassemia [100]. Also, it has been widely used in mutation detection of Janus kinase 2 (JAK2) in leukemia and mutation detection in HIV. One limitation of AS-PCR is that it needs to be designed for each specific mutation, and thus, it cannot be used for identifying or discovering novel mutations or detection of chromosomal abnormalities. The main limitation is related to the high amplification efficiency of PCR. In qualitative set ups AS-PCR is extremely prone for contamination errors. When properly used, AS-PCR is, however, inexpensive, fast, reliable accurate and rapid for detection of single or a limited number of SNPs, whereas utilizing AS-PCR for detection of multiple SNPs would require a complex and time-consuming analytical procedure [100]. Currently AS-PCR has been substituted by real-time and digital droplet PCR (ddPCR) technologies in most clinical applications.

Real-time PCR

The original PCR methods used end point detection techniques, such as gel electrophoresis, for visualization of amplification products. These techniques are performed at the plateau phase of amplification and as a result, they allow qualitative, or at best, semiquantitative detection of amplification products. The first quantitative PCR techniques were based on co-amplification of synthetic or cloned internal standard templates that allowed for relatively accurate quantification of the PCR products. These techniques were mainly hampered by the requirement of including a separate internal standard for specific target amplicon. Most early techniques also required post-amplification handling of the PCR-product, causing a risk of contamination. Real-time PCR (or quantitative PCR – qPCR) has since, become the most effective and popular method for quantitative PCR. Real-time PCR is based on quantification of the PCR products in real-time during the exponential phase of amplification [96]. Real-time PCR is based on fluorescence kinetics to detect the PCR product and to assess the amount of the original DNA or cDNA template present in the reaction. The real-time PCR instrument measures the fluorescence intensity signals in the reaction tubes at every PCR cycle by detecting light emission released from fluorescent probes or double stranded DNA-binding dyes during the PCR reaction. Real-time PCR is a sensitive, reproducible, and accurate technique. The analytical procedure is relatively easy, requiring no post-PCR manipulation. [96]. qPCR is considered as the golden standard method for quantitative analysis of nucleic acids. The technique is widely used in research, as well as, in a wide range of clinical diagnostic applications [96].

Digital PCR

Digital droplet polymerase chain reaction (ddPCR) has emerged as a precise technique for absolute quantification of DNA and RNA template, especially when they occur at low concentrations. The

main difference between ddPCR and real-time PCR is that ddPCR can measure the absolute template molecule amounts of nucleic acids with a higher precision [101]. In ddPCR, the amplification is separated into many partitions, or droplets, each containing only one, or no template DNA molecules, the reagents, and substrates for the amplification reaction. The reaction proceeds independently in each partition. As there are only one or zero template molecules per droplet the result is digital. Another benefit of ddPCR is that DNA quantification in partitions takes please in closed containers that are not opened during analysis, which minimizes the risk of sample contamination by amplification products [102]. The technique is useful for studying variations and point mutations, which require precise quantification of the copy number of the template nucleic acids in the samples [103, 104].

3.2. Sequencing and next-generation sequencing

DNA sequencing

DNA sequencing is a process of determining the order of the four nucleotides (A, T, G, C) in a specific DNA sequence. It has been used to determine the sequence of individual genes, or full chromosomes, or entire genomes of an organism. The knowledge of the DNA sequence is vitally important in many areas of biological and medical research, including forensic sciences. Identification of mutations related to malignant diseases by comparing to the DNA sequence in cancer cells and healthy cells from the same individual, provides a possibility for molecular diagnosis of the somatic genetic variations making individually tailored treatment in patients with cancer possible [103, 105, 106].

The first generation of DNA sequencing is known as Sanger sequencing. Sanger sequencing utilizes gel- or capillary electrophoresis to identify sequencing products generated in reactions specific for each of the four nucleotides, based on length. It has been widely used for determining DNA sequences for several decades. Currently Sanger sequencing still has a place in applications requiring long sequence reads of >700 nucleotides and for validation of new sequencing methods [107]. Sanger sequencing is widely used for verification of diagnostic mutations found using next generation sequencing.

Next-generation sequencing

Next-generation sequencing (NGS), or Massive Parallel Sequencing has largely substituted Sanger sequencing nowadays, because of its enormous capacity, making large-scale and automated analysis possible. NGS performs the sequencing process with hundreds of millions of small fragments of DNA in parallel, and provides accurate sequence data [108]. NGS has enabled whole genome sequencing (WGS) and at writing, multiple human genomes can be sequenced in one run within 30 hours using NGS [108, 109]. Currently, the main limitations to NGS, are determined by computing capacity and storage, and especially the personnel expertise required for comprehensive analysis and interpretation of the sequencing data [108].

RNA sequencing

RNA sequencing (RNA-seq) is a particular sequencing technique which uses NGS to identify the RNA in a biological sample [110, 111]. RNA is usually converted to cDNA before preparing library for the sequencing step, or RNA can be directly sequenced in a massively parallel manner [111-113]. A major advantage of RNA-seq is the possibility to analyse the gene expression of individual cells by single cell RNA sequencing (scRNA-seq) [107]. Previous, probe-based techniques used for analysing gene expression, such as microarrays, analyse RNA profiles in mixed cell populations. These techniques cannot unveil the differences in RNA expression between individual cells within these cell populations [114, 115] In addition, RNA-seq circumvents the need for prior knowledge of the sequence of the target RNA, which is an inherent limitation of any probe-based technique [116].

3.3. Other techniques

Microarrays

Microarrays is a probe-based technique used for detection of the expression of thousands of genes at the same time in a two-dimensional array chip. Microarrays are composed of thousands of tiny spots printed on microscopic slides. Each spot contains a known single stranded DNA or RNA sequence that act as probes for detection of gene expression. DNA, or cDNA that has been transcribed from RNA is labelled with fluorescent probes of difference colours. Usually DNA/cDNA from experimental samples is labelled with red fluorescent dye, whereas the reference DNA/cDNA is labelled with green, fluorescent dye. Following hybridization. At the hybridization step the molecules compete for binding to the probe, and the assay is based on that the relative amounts of the bound molecules represent the relative amounts of the molecules in the liquid phase. After hybridization and washes the microscopic slide is scanned and fluorescence intensities of the dyes is measured for each probe spot. This allows for assessment of the relative amounts of that fragment of the experimental and reference samples [117]. Today, microarrays can use both DNA and RNA probes for detection. Microarrays are routinely used in molecular diagnostics of large structural variations of multiple malignant and non-malignant diseases, as well as diagnostics of prenatal and developmental disorders

Comparative genomic hybridisation (CGH) is the predecessor of Microarrays and it is a molecular cytogenetic technique that can be used for detection of chromosomal copy number or chromosomal structural variations [118]. It uses the same two-colour competitive hybridization presented above, but instead of oligonucleotide probes the samples are hybridized to chromosomes fixed onto the surface of a microscopic slide. Originally it was developed for evaluation of the differences between the chromosomes in tumour and a normal tissues [119]. It increases the resolution compared to traditional techniques, such as fluorescence in situ hybridization (FISH) [120]. As the result of development inexpensive and efficient methods for synthesis of oligonucleotide probes on the surface of a microscopic slide, CGH has been replaced by Microarrays, or so-called CGH arrays.

Fluorescence in situ hybridization (FISH)

Fluorescence in situ hybridization (FISH) is a cytogenetic technique, which uses binding of target specific fluorescent probes to chromosomes of cells fixed on the surface of a microscopic slide. The binding of the test probe is detected using a fluorescence microscope. FISH is used to detect and locate a specific DNA sequence rearrangement in chromosomes of individual cells [120]. FISH can detect smaller chromosomal changes than standard cytogenetic methods such as in karyotyping [121]. FISH is diagnostic tool for some chromosomal abnormalities, such as Prader-Willi syndrome, Angelman syndrome, 22q13 deletion syndrome, and Down syndrome. Moreover, FISH can also be used for cancer diagnostics. FISH is the only tool in which DNA is not isolated but studied *in situ*, without pre-amplification.

Blotting

Blotting is a molecular method in which DNA (Southern blotting), or RNA (Northern Blotting) is transferred onto a membrane that typically consists of nitrocellulose, polyvinylidene fluoride or nylon. After blotting, the products are visualized by binding of labelled probes. Originally, probes were cloned DNA fragments that were labelled with radioisotopes.

AIMS OF THE STUDY

The general aim of this study was to develop and apply a novel technique for ultrasensitive quantification of expressed mutations to evaluate the tissue expression of KRAS and BRAF mutations as a diagnostic and/or prognostic marker in certain MAPK/ERK-driven cancers. Colorectal cancer and thyroid cancer, as well as a potential pre-malignant condition, esophageal atresia, was used as models for this purpose.

The specific aims of this study were:

1. To develop a novel mutation detection method with high sensitivity and specificity for determination of the levels of expressed mutations at RNA level.
2. To study the association of expressed KRAS and BRAF mutant mRNA levels in colorectal cancer tissue with diagnostic and prognostic variables.
3. To study the putative role of RNA expression of BRAF mutations as diagnostic markers in thyroid cancer.
4. To study the putative role of RNA expression of KRAS and BRAF mutations as early markers for malignant transformation in esophageal atresia.

MATERIALS AND METHODS

1. MUTATION DETECTION TECHNIQUE DEVELOPMENT

1.1. RNA templates

RNA templates used for technique development were generated by in-vitro transcription using DNA templates with sequences corresponding to the wildtype and different mutated variants of the KRAS and BRAF genes. DNA templates for in-vitro transcription were generated using PCR amplification of synthetic DNA oligonucleotides. PCR amplification was performed with Phusion High-Fidelity DNA polymerase (Thermo Scientific) and synthetic DNA oligonucleotides were obtained from TAG Copenhagen A/S. Each oligonucleotide was about 100 nucleotides in length, including 20 nucleotides of T7 promoter's sequence (in bold) at the 5' end (**Table 1**).

Table 1: Sequences of DNA oligonucleotides used as templates to synthesize different RNA variants (Original article I, Ho, T. H., Dang, K. X., 2015) [122]

Oligos	**Sequences with variant nucleotide in red** (5'-3')
KRAS wildtype	**TAATACGACTCACTATAGGG**ATGACTGAATATAAACTTGTGGTAGTTGGAGC TGGTGGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCA
KRAS G12D (GGT>GAT)	**TAATACGACTCACTATAGGG**ATGACTGAATATAAACTTGTGGTAGTTGGAGC TGATGGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCA
KRAS G12A (GGT>GCT)	**TAATACGACTCACTATAGGG**ATGACTGAATATAAACTTGTGGTAGTTGGAGC TGCTGGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCA
KRAS G12V (GGT>CTT)	**TAATACGACTCACTATAGGG**ATGACTGAATATAAACTTGTGGTAGTTGGAGC TGTTGGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCA
KRAS G12S (GGT>AGT)	**TAATACGACTCACTATAGGG**ATGACTGAATATAAACTTGTGGTAGTTGGAGC TAGTGGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCA
KRAS G12R (GGT>CGT)	**TAATACGACTCACTATAGGG**ATGACTGAATATAAACTTGTGGTAGTTGGAGC TCGTGGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCA
KRAS G12C (GGT>TGT)	**TAATACGACTCACTATAGGG**ATGACTGAATATAAACTTGTGGTAGTTGGAGC TTGTGGCGTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCA
BRAF wildtype	**TAATACGACTCACTATAGGG**TGAAGACCTCACAGTAAAAATAGGTGATTTTG GTCTAGCTACAGTGAAATCTCGATGGAGTGGGTCCCATCAGTTTGAAC
BRAF V600E (GTG>GAG)	**TAATACGACTCACTATAGGG**TGAAGACCTCACAGTAAAAATAGGTGATTTTG GTCTAGCTACAGAGAAATCTCGATGGAGTGGGTCCCATCAGTTTGAAC

The DNA templates were used to synthesize RNAs by in vitro transcription with AmpliScribe T7, T3, and SP6 High Yield Transcription Kits (Epicentre Biotechnologies) according to manufacturer's instruction. The concentrations of resulting RNA samples were quantified using a NanoVue spectrophotometer (GE Healthcare, Waskesha, WI), and the copy numbers of the different RNA variants were verified using quantitative RT-PCR (Tetro cDNA synthesis kit and SensiFAST SYBR No-ROX Kit, Bioline). The primers for these RT-PCR assays were obtained from TAG Copenhagen A/S. The primer sequences are listed in **Table 2**.

Table 2: Primer sequences used in RT-PCR assays for quantification of total KRAS and BRAF RNA transcripts (Original article I, Ho, T. H., Dang, K. X., 2015) [122]

RT-PCR assays	**Primers**	**Concentration**	**Sequences** (5'-3')
KRAS	Reverse transcription primer	0.5 μM	AAATGATTCTGAATTAGCTGT
	PCR forward primer	0.5 μM	GACTGAATATAAACTTGTGGTAGTTG
	PCR reverse primer	0.5 μM	TAGCTGTATCGTCAAGGC
BRAF	Reverse transcription primer	0.5 μM	ACTGTTCAAACTGATGGGACCCAC
	PCR forward primer	0.5 μM	AGACCTCACAGTAAAAATAGGTGA
	PCR reverse primer	0.5 μM	GACCCACTCCATCGAGATTTC

The RNA samples corresponding to KRAS and BRAF wildtype transcript sequences, as well as six possible KRAS codon 12 variants, and the BRAF V600E (GTG>GAG) mutation, were used for the assay development and the determination of the selectivity of given assays. Human RNA samples were used to demonstrate a proof-of-principle for analysis of expressed mutations using the ExBP-RT assay. Human RNA samples were extracted from formalin-fixed paraffin embedded (FFPE) samples of colorectal cancer tumour tissue using phenol-chloroform extraction [123]. The use of clinical samples for this purpose was approved by the institutional Ethics Committee. All RNA samples were quantified with a NanoVue spectrophotometer (GE Healthcare, Waskesha, WI) and diluted to 500 ng/μl in diethylpyrocarbonate (DEPC) H_2O, before the allele-specific reverse transcription reaction.

1.2. Reaction conditions of the ExBP-RT assay

For each analysed mutation, a mutation-specific primer was designed to target to the mutant RNA and a wildtype-specific blocking probe was designed to target to the wildtype RNA (**Table 3**). The mutation-specific primer has a 5'-prime tail which generated a priming site of non-related sequence for the subsequent amplification reactions. Both the mutation-specific primer and the blocking probe were included in each reverse transcription reaction. All components of the cDNA synthesis reactions (except the enzyme reverse transcriptase) were assembled according to the manufacturer's instruction to a 10 μL reaction volume. The reactions were incubated at 65°C for 5 minutes, then cooled down to 50°C before adding reverse transcriptase enzyme (Tetro Reverse Transcriptase, Bioline, London, UK). Subsequently, the reaction temperature was decreased by 1°C every 1 minute from 50°C to 37°C. At the end, the reaction temperature increased to 85°C for 5 minutes to inactivate the enzyme. The resulting cDNA products were stored at -20°C for later analysis.

The KRAS G12D mutation detection assay used a non-extendable oligo which hybridizes to a region downstream of the priming site on the RNA template. This oligo prevented primer extension resulting from nonspecific priming of allele-specific RT primers to a wrong locus

downstream of the expected priming site. This oligo's sequence was: 5'-GAATTAGCTGTATCGTCAAGGCACTAAAAAA-3'.

Along with single-plex ExBP-RT for each specific mutation, we included a multiplex ExBP-RT assay which contains six different mutation-specific primers targeting six possible KRAS mutations at codon 12 and a common ExBP targeting the wildtype KRAS transcript (sequences of primers and probe in **Table 3**). The concentrations of primers and probes is the same as those of primers and probes in the single-plex ExBP-RT assay. Due to the presence of many primers and probe in a single reaction of multiplex ExBP-RT, the optimal concentration of Mg^{2+} ion has been adjusted and optimized to 10 mM. In addition, the unbound primers and nucleotides were degraded by incubating at 37°C for 30 min with 10 units of Exonuclease I (Thermo Scientific) and 1 unit of Thermosensitive Alkaline Phosphatase (Thermo Scientific). The resulting cDNA products were then used in the quantitative PCR step.

Table 3: Primer and probe sequences for different ExBP-RT assays (Original article I, Ho, T. H., Dang, K. X., 2015) [122]

ExBP-RT assays	**Primers and probes**	**Sequences** (The engineering 5'-tail sequences **in bold**)	**Conc**
KRAS G12D (GGT>G**A**T)	Mutation-specific primer	**GCCGATCAGACGACGACTATTATT**CCATCAGCT	2 µM
	Wildtype-specific blocking probe	GCCACCAGCT	4 µM
KRAS G12A (GGT>G**C**T)	Mutation-specific	**GCGCCGATCAGACGACGACTTATT**CCAGCAGCT	2 µM
	Wildtype-specific	GCCACCAGCT	4 µM
KRAS G12V (GGT>C**T**T)	Mutation-specific primer	**GCCGATCAGACGACGACTATTATT**CCAACAGCT	2 µM
	Wildtype-specific blocking probe	GCCACCAGCT	4 µM
KRAS G12S (GGT>**A**GT)	Mutation-specific primer	**GCCGATCAGACGACGACTATTATT**CCACTAGCT	2 µM
	Wildtype-specific blocking probe	GCCACCAGCT	4 µM
KRAS G12R (GGT>**C**GT)	Mutation-specific primer	**GCCGATCAGACGACGACTATTATT**CCACGAGCT	2 µM
	Wildtype-specific blocking probe	GCCACCAGCT	4 µM
KRAS G12C (GGT>**T**GT)	Mutation-specific primer	**GCCGATCAGACGACGACTATTATT**CCACAAGCT	2 µM
	Wildtype-specific blocking probe	GCCACCAGCT	4 µM
BRAF V600E (GTG>G**A**G)	Mutation-specific primer	**GCCGATCAGACGACGACTATTATT**GATTTCTCTG TAG	1 µM
	Wildtype-specific blocking probe	BRAF-INERT: AGATTTCACTGTAG	4 µM
		BRAF-PO4: AGATTTCACTGTAG- PO4	4 µM
		BRAF-Atail: AGATTTCACTGTAG-AAAAAA	4 µM

1.3. Quantitative PCR reaction conditions

One micro litre aliquots of each cDNA synthesis product were used as templates in the following 10 µL quantitative PCR (qPCR) reactions. The detection reagents were SensiFAST SYBR No-ROX kit (Bioline) and SensiFAST Probe No-ROX kit (Bioline) for probe-based detection. The qPCR thermal conditions were as follows: Initial incubation at 95°C for 2 min, followed by 42 cycles of 95°C for 5 sec, 63°C for 20 sec and 72°C for 10 sec. In multiplex ExBP-RT, QuantiTect SYBR PCR kits (Qiagen) with thermal condition of firstly 95°C for 15 min, then following 45 cycles of 94°C for 15 sec and 60°C for 45 sec was used. The primer and probe sequences for each qPCR assay are listed in **Table 4**. All qPCR assays were performed on a LightCycler 480 II Real-Time PCR Instrument (Roche Diagnostics) using a 384-well thermal block. Following SYBR-based qPCR, the specificity of the amplification products was verified by melting curve analysis. Amplification efficiencies of qPCR assays used in this study were determined to be close to 100%. All reactions were run in duplicate or higher replication numbers where so specified. All replicates went through both ExBP-RT and qPCR steps.

Table 4: Primer and probe sequences for PCR step of different ExBP-RT assays (Original article I, Ho, T. H., Dang, K. X., 2015) [122]

ExBP-RT assays	**PCR primers**	**Conc**	**Sequences** (5'-3')
KRAS G12D probe-based qPCR	PCR forward primer	0.6 µM	AGGCCTGCTGAAAATGACTG
	PCR reverse primer	0.6 µM	CGATCAGACGACGAC
	Probe	0.4 µM	FAM-ATT+AT+TCCA+TCA+gC+TCC-BHQ1 (N+ stands for LNA)
KRAS mutation SYBR Green I qPCR	PCR forward primer	0.2 µM	GACTGAATATAAACTTGTGGTAGTTG
	PCR reverse primer	0.2 µM	CGATCAGACGACGAC
BRAF mutation SYBR Green I qPCR	PCR forward primer	0.5 µM	TGAAGACCTCACAGTAAA
	PCR reverse primer	0.5 µM	CGATCAGACGACGAC
KRAS mutations (Multiplex ExBP-RT)	PCR forward primer	0.3 µM	CCTGCTGAAAATGACTGAA
	PCR reverse primer	0.3 µM	CGATCAGACGACGAC
Total KRAS transcript (Multiplex ExBP-RT)	PCR forward primer	0.3 µM	CCTGCTGAAAATGACTGAA
	PCR reverse primer	0.3 µM	GCCACCAGCTCCAACTACCACAA

1.4. Data analysis

Threshold cycle (Ct) values for qPCR were calculated automatically using the default second derivative maximum method, which is built in the LightCycler® 480 II system (Roche Diagnostics). The selectivity of each ExBP-RT or other assays for detecting mutant RNA transcripts among a surplus of wildtype transcripts was determined by comparing products formed in the first reaction containing mismatched template (wildtype RNA) with those formed in the second reaction containing the same copy number (10^7 copies) of matched templates (mutant RNA). The ratio between the Ct value of the reaction measuring the amount of the wildtype cDNA and the Ct-value of the reaction measuring the amount of the mutant cDNA ($\Delta Ct_{wt\text{-}mt} = Ct_{wildtype} - Ct_{mutant}$), was used to assess the relative RNA level of the mutant allele. The sensitivity of each

ExBP-RT assay, expressed as percentage, was calculated as $2^{-\Delta Ct}$ x 100%, which correspond to the lowest fraction of mutant transcripts to be detected as a distinct signal from the background signal derived from the wildtype template.

2. DETECTION OF EXPRESSED KRAS AND BRAS MUTATIONS IN GASTROINTESTINAL AND THYROID SAMPLES

2.1. Patients and clinical data

Colorectal cancer (CRC)

All patients included in the study cohort underwent surgery for histologically confirmed CRC in the Department of Surgery, Helsinki University Hospital, Finland, between 1987 and 2003. In total, archived tissue samples were available for 571 patients (**Table 5**). The Surgical Ethics Committee of Helsinki University Hospital approved the study protocol. The National Supervisory Authority of Welfare and Health granted permission to use the archived tissue samples without necessitating individual informed consent for this retrospective study. Clinical data were collected from patient records, survival data were collected from the Finnish Population Register Centre and cause of death data were collected from Statistics Finland.

Table 5: Patient characteristics among patients with colorectal cancer (n = 571)

		n	%
Age	<65	231	40
	≥65	340	60
Gender	Female	264	46
	Male	307	54
Histology	Adeno	513	90
	Mucinous	58	10
Location	Colon	381	67
	Rectum	190	33
Side	Right	201	35
	Left	369	65
	NA	1	0
TNM stage	I	74	13
	II	211	37
	III	166	29
	IV	120	21
Differentiation grade	1	28	5
	2	392	69
	3	109	19
	4	21	4
	NA	21	4

Thyroid cancer (TC)

FFPE tissue samples from 62 patients were obtained from the Department of Pathology, 103 Military Hospital, Hanoi, Vietnam (**Table 6**). Multiple 10 µm-thickness sections containing 10 mg of FFPE tissue were collected and deparaffinized by mineral oil before extraction of nucleic acids. The use of the clinical samples for this study was approved by the Ethics Committee of the Vietnam Military Medical University according to the Declaration of Helsinki. Consent was provided by all participants orally and their specimens could be stored in the hospital database and used in research through a written document. Patient's records were anonymized and contained no identifiable traits.

Table 6: Clinicopathologic parameters in patients with thyroid diseases (n = 62) (Original article III, Dang, K. X., Tran, T. V., 2020) [124]

Clinicopathologic parameters		Frequencies	
		Number	Percentage (%)
Sex	Male	7	11.3
	Female	55	88.7
Histology of malignant tumours	Papillary	24	75.0
	Follicular	6	18.8
	Mixed Papillary – Follicular variant	1	3.1
	Thyroid Adenocarcinoma	1	3.1
Histology of benign tumours	Nontoxic single thyroid nodule	9	30.0
	Benign neoplasm of thyroid gland	20	66.7
	Basedow with euthyroid phase stage	1	3.1

The use of clinical diagnostic tissue samples for this purpose was approved by the Surgical Ethics Committee of Helsinki University Hospital and the National Supervisory Authority of Welfare and Health, and samples were collected from the archives of the Department of Pathology, Helsinki University Hospital.

Esophageal atresia (EA)

During 2001-2004, 112 endoscopic biopsies were collected prospectively from 61 patients treated for EA in early childhood (at the Hospital for Children and Adolescents, Helsinki University Central Hospital). Sex, type, histology and other characteristics of this cohort are described on **Table 7**. The age profile is on **Table 8**. Informed consent was received from all patients participating in this study in accordance with the Helsinki declaration. The study was approved by the ethics committee of the Hospital for Children and Adolescents, University of Helsinki, Finland.

Table 7: Characteristics of esophageal atresia patients (n = 61). The long-gap disease is defined by a distance between the upper and lower pouches of more than 3 cm or at least two vertebral bodies on plain radiography; Biopsies were taken 2 cm above the esophagogastric junction.

		Patients	Percentage	Total
Sex	Male	35	57.4	61
	Female	26	42.6	
Type	Tracheoesophageal fistula (TEF)	1	1.6	61
	Distal TEF	55	90.2	
	Proximal and distal TEFs	3	4.9	
	H-type TEF	2	3.3	
Histology	Esophagitis	5	25	20
	Gastric metaplasia	10	50	
	Intestinal metaplasia	5	25	
Misc.	Long-gap disease	6		
	Tracheomalacia	10		
	Re-operated due to a fistula	6		
	dilatations of the esophageal anastomosis	59		
	Surgical resection of a stricture	4		
	Fundoplication	10		

Table 8: The age profiles of esophageal atresia patients

	Min	Max	Mean	Median
Age at biopsy	21	56	35.6	32.7

2.2. RNA samples

Colorectal cancer

RNA was extracted from formalin-fixed paraffin-embedded (FFPE) samples from CRC patients. In total, 775 patient samples were available for this study. RNA was extracted using phenol–chloroform extraction [123] and stored at -80°C. All RNA samples were quantified with a NanoVue spectrophotometer (GE Healthcare, Waukesha, Wisconsin, USA), then diluted to 100 ng/μL in diethyl pyro-carbonate (DEPC) H_2O.

Thyroid cancer

RNA was extracted using GenElute FFPE RNA Purification Kit (Sigma-Aldrich), and DNA was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen, Germany), according to the manufacturers' instructions. The nucleic acid concentration was determined using an ND-1000 spectrophotometer (NanoDrop, Walmington, DE). *In-vitro* transcribed RNA of the mutated BRAF

V600E variant (mutant cRNA) and wildtype BRAF (wildtype cRNA) was utilized for determination of the sensitivity of BRAF V600E RNA-based mutation assay.

Esophageal atresia

Endoscopic biopsy samples were collected in RNA*later* (Sigma-Aldrich) and stored at 4°C until extraction within one week from sampling. RNA samples were extracted from fresh frozen endoscopic tissue biopsies from EA patients using phenol-chloroform extraction [123] then stored at -80°C. Before the extendable blocking probe reverse transcription (ExBP-RT) reaction, all RNA samples were quantified with a NanoVue spectrophotometer (GE Healthcare, Waskesha, WI) then diluted to 100 ng/µl in Diethylpyrocarbonate (DEPC) H_2O.

RNA controls

RNA extracted from A549, Lovo and Colo205 cell lines was used as either positive or negative controls in experiments (**Table 9**). All control RNAs were extracted from cell cultures using RNA/DNA purification Kit (Norgen Biotek) then quantified with a NanoVue spectrophotometer and diluted to 100 ng/µl in DEPC H_2O before using.

Table 9: Cell line RNA used as positive controls. (Original article II, Dang, K. X., 2018) [125]

Cell line	Tissue	Mutation	KRAS Codon 12 mutation analysis	KRAS Codon 13 mutation analysis	BRAF mutation analysis
A549	Lung	KRAS - G12R	Mutant control	-	Wild-type control
Lovo	Colon	KRAS - G13D	-	Mutant control	-
Colo205	Colon	BRAF - V600E	Wild-type control	Wild-type control	Mutant control

2.3. Mutation detection assays for clinical samples

ExBP-RT–based mutation detection assays

Table 10: Primer, blocking probe for ExBP-RT assays of mutant KRAS codon 13 (Locked Nucleic Acid (LNA) = (+A), (+G), (+C), (+T)) (Original article III, Dang, K. X., Tran, T. V., 2020) [124]

Primers and probes	Sequences (The engineering 5'-tail sequences **in bold**)	Concentrations
KRAS codon 13 Mutation-specific primer		
KRAS G13D (GGC>G**A**C)	5'-**CGATCAGACGACGACTATTAT**TACG(+T)CACC-3'	0.5 µM
KRAS codon 13 Wildtype-specific blocking probe		
KRAS13-INERT	5'-CTACGCCACCA-3'	4 µM

The multiplex ExBP-RT mutation detection assay included six different common KRAS mutations at codon 12 (G12D, G12A, G12V, G12S, G12A, G12C); one KRAS mutation at codon 13

(G13D); and one BRAF mutation (V600E). The principles of ExBP-RT assays and reaction set-up procedures for multiplex detection of six KRAS mutations at codon 12 and one BRAF mutation (V600E) were similar to those described in the mutation detection technique section (primers and probe are listed in **Table 3**). ExBP-RT primers and probe for detection of KRAS mutation at codon 13 (G13D) are listed in **Table 10**.

Detection and Quantification of Expressed Mutations

Table 11: Primer and probe sequences for qPCR step of different ExBP-RT assays (Locked Nucleic Acid (LNA) = (+A), (+G), (+C), (+T); Inosine = i; 6-carboxyfluorescein: FAM; Black Hole Quenchers: BHQ) (Original article III, Dang, K. X., Tran, T. V., 2020) [124]

Primers and probes	**Sequences** (5' – 3')	**Concentrations**
Mutant KRAS codon 12 assays		
KRAS Forward primer	5'-CCTGCTGAAAATGACTGAA-3'	0.5 µM
Common Reverse primer	5'-CGATCAGACGACGAC-3'	0.5 µM
KRAS12-Probe	FAM-AT(+T)A(+T)T(+C)C(+A)ii(+A)G(+C)TCC-BHQ1	0.1 µM
Mutant KRAS codon 13 assays		
KRAS Forward primer	5'-CCTGCTGAAAATGACTGAA-3'	0.5 µM
Common Reverse primer	5'-CGATCAGACGACGAC-3'	0.5 µM
KRAS13-Probe	FAM-AGC(+T)GG(+T)GA(+C)G(+T)AA(+T)AAT-BHQ1	0.1 µM
Total-KRAS assays		
KRAS Forward primer	5'-CCTGCTGAAAATGACTGAA-3'	1.5 µM
Total KRAS Reverse primer	5'-GCCACCAGCTCCAACTACCACAA-3'	1.5 µM
Mutant BRAF assays		
BRAF Forward primer	5'-AGACCTCACAGTAAAAATAGGTGA-3'	0.5 µM
Common Reverse primer	5'-CGATCAGACGACGAC-3'	0.5 µM
BRAF-Probe	FAM-TTC(+T)CT(+G)TA(+G)CT(+A)GACCAA-BHQ1	0.1 µM
Total-BRAF assays		
Total BRAF Forward primer	5'-CATGAAGACCTCACAGTAAA-3'	1.5 µM
Total BRAF Reverse primer	5'-GATTTCACTGTAGCTAGACC-3'	1.5 µM

Using cDNA products of the ExBP-RT assays as template, real-time PCR amplification was performed to detect/quantify expressed mutations of KRAS and BRAF genes. QuantiTect Probe PCR Kits (QIAGEN) were used for these probe-based real-time PCR assays according to the manufacturer's instructions in a 10 µL reaction volume. The Taqman probes for simultaneous detection of six different KRAS mutations at codon 12 were designed to contain 2 universally binding inosine nucleotides that allows for targeting the variant nucleotides of different types of mutation. A common reverse primer was designed to target the 5'-prime tail of all mutation-specific

ExBP-RT products. The expression levels of total KRAS and total BRAF genes (both mutant and wildtype) were also determined in each sample for normalization using QuantiTect SYBR® Green PCR Kits (QIAGEN) according to the manufacturer's instructions in a 10 μL volume. The sequences and concentration of qPCR primers and probes are provided in **Table 11**. The same thermocycling conditions were used for both probe-based and SYBR-based qPCR: firstly 95°C for 15 min, then following 45 cycles of 94°C for 10 sec, 60°C for 45 sec. Following SYBR-based qPCR, the specificity of the amplification products was always verified by melting curve analysis. All qPCR assays were run on a LightCycler 480 II Real-Time PCR Instrument (Roche Diagnostics Oy, Finland) with 384-well white-plate. All mutation controls, wildtype controls and H_2O controls of each experiment were checked to verify the correction of results in both ExBP-RT, qPCR assays and avoid the problem of contamination.

2.4. Data and statistical analysis

Quantitative PCR (qPCR) results were calculated by threshold cycle (Ct) values automatically using the Absolute Quantification Analysis with the Fit Points Method, which is built in the LightCycler® 480 II system (Roche Diagnostics Oy, Finland). This allows for manually setting a threshold for discarding uninformative background noise.

All results were presented as the mean and standard deviation (SD), the median and interquartile range (IQR) or the number of patients and percentage. We tested the differences in continuous variables between groups using the non-parametric Mann–Whitney test, whereas we analysed dichotomous or ordinal variables using the Fisher's exact test or the linear-by-linear association test. The Kaplan–Meier method and the Cox proportional hazard model were used to analyse survival data. The multivariate model was adjusted for age, gender, and tumour location (rectum/colon). Interactions were considered using the Bonferroni correction for multivariate analysis. The Cox model assumption of constant hazard ratio over time was tested by plotting the scaled Schoenfeld residuals with time and testing the relationship between residuals and time. No significant deviation from the assumption was detected. Statistical analyses were performed with R, version 3.4.3 (Foundation for Statistical Computing, Vienna, Austria, survival package, https://CRAN.R-project.org/package=survival) and SPSS, version 24 (IBM, New York, USA).

RESULTS

1. ESTABLISHMENT OF EXTENDABLE BLOCKING PROBE – REVERSE TRANSCRIPTION AS A NOVEL MUTATION DETECTION METHOD

Establishing the ExBP-RT technique

Hypothesis

We hypothesized that the RNA expression of specific mutations in tumour tissue, might potentially have additional diagnostic and prognostic value compare to determination of mutational status in DNA alone. Since tumour tissue typically contains a mixture of many different cell types and not all cells harbour the mutations, the mutated RNA may be represented at a much higher abundance than the DNA counterpart. There's a need for a quantitative detection technology with a high capacity (selectivity) for distinguishing the mutated RNAs from the wildtype RNA alleles. We developed a new technique using reverse transcription for direct conversion of RNA variants with minor differences, such as single-base substitutions or point mutations, into easily distinguishable allele-specific cDNAs that can be separately amplified by PCR. This technique was named Extendable Blocking Probe Reverse Transcription (ExBP-RT).

Reverse transcription

In the first step, the RNA template is converted to cDNA in a reverse transcription reaction. The reaction contains a mutation-specific primer and also a wildtype-specific competitive blocking probe which is extendable, thus lacking a dideoxy 3'-terminal nucleotide, which is typically used in many PCR-based techniques in order to prevent the blocking probes from being extended by the DNA polymerase (**Figure 4-A**). Both primer and blocking probe have a priming sequence that is fully complementary to a specific RNA variant (a mutation-specific primer to the mutant RNA; and a wildtype-specific competitive blocking probe to the wildtype RNA). But they also form a mismatch with the alternative RNA variant at the variation site. The primer and blocking probe were designed to be short in length (about 10 nucleotides) in order to maximize the discrimination between a perfect match and a mismatch during the reverse transcription reaction. The mutation-specific primer additionally contains an engineered sequence of non-related DNA at the 5'-tail, which allows for selective amplification of the mutation-specific cDNA in the subsequent PCR step.

Thermal conditions

To maximize the correct annealing of primers and probes at their optimal temperature (higher temperature) and minimize mis-priming at lower temperatures, we utilized a hot-start protocol in reverse transcription. The temperature of reaction was cooled down slowly (in 1°C increments) towards the optimal temperature for primer annealing. At the optimal temperature, the blocking probe perfectly hybridizes to wildtype RNA template and extends to form cDNA. Simultaneously, the mutation-specific primers bind to the mutant RNA template and extends to form the mutant cDNA. With a sufficient concentration of blocking probe, all RNA of wild-type alleles will be completely extended to cDNA. The newly formed wild-type cDNA, resulting from extension of

the extendable blocking probe, remains bound to the wildtype RNA template and effectively prevents mis-priming by the mutation-specific primer when the reaction temperature is further lowered to a level where mis-priming would be likely to occur. The wildtype cDNA/RNA hybrids thus, efficiently prevent the much shorter mutation-specific primers from binding to the wild-type RNA template. In this way the wildtype template is effectively neutralized with the extendable blocking probes and mis-priming is by the mutant-specific primer is efficiently suppressed. Since the mutant-specific primer also binds to the specific mutation site and extends the RNA template in an early stage of the reverse transcription reaction, mis-priming of the extendable blocking probe to the mutant RNA template is inhibited exactly by the same mechanism.

PCR amplification

The mutation-specific primer used in the reverse transcription step was designed to have a unique unrelated sequence at its 5'-tail. This signature sequence is incorporated in the mutation-specific cDNA, generating a unique downstream priming site in the subsequent mutation-specific PCR amplicon. In the PCR reaction, only the mutant cDNA product has priming-site for the reverse primer (**Figure 4-B**), which will be amplified and detected. The wildtype cDNA product, on the other hand, lacks the primer-binding site for the reverse PCR primer and will thus, not be amplified.

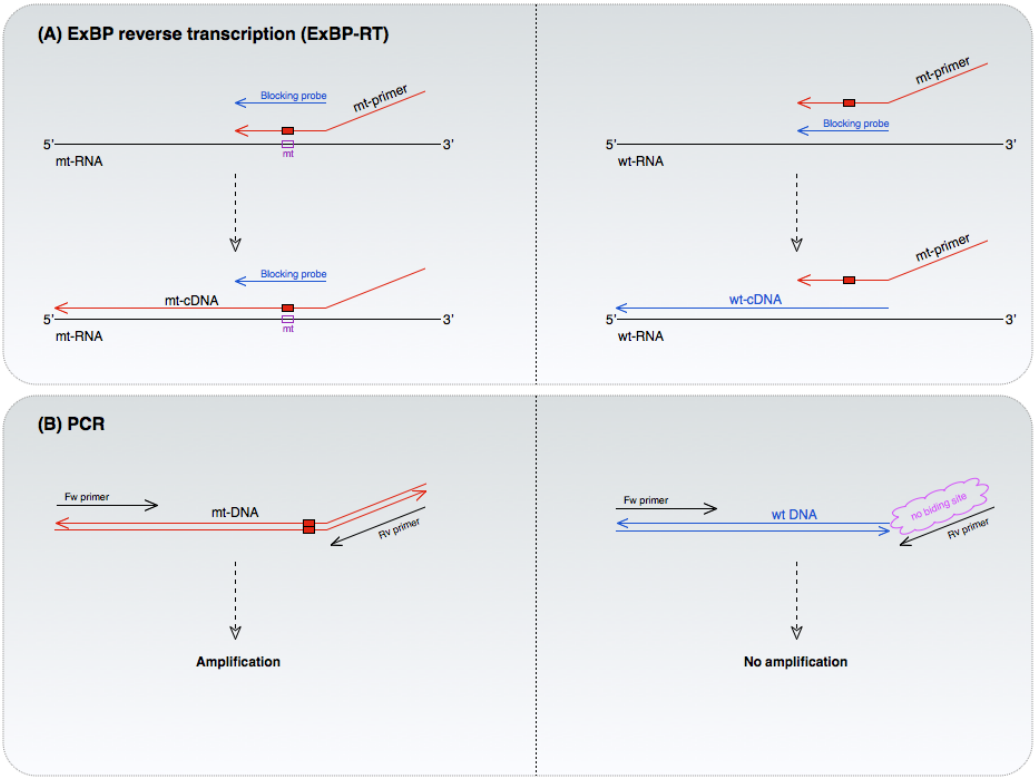


Figure 4: Principle of the allele-specific reverse transcription (ExBP-RT) assay. The analytical procedure includes two steps: (A, step1) reverse transcription with a mutation-specific RT primer and an extendable competitive blocking probe (B, step 2) selective PCR amplification and detection/quantification. While the mutation-specific primer, which contains a nucleotide tail of unrelated sequence, generates a PCR-amplifiable cDNA product, the competitive blocking probe without tail produces a cDNA lacking primer-binding site for the reverse PCR primer. (Original article I, Ho, T. H., Dang, K. X., 2015) [122].

The selectivity of ExBP-RT technique

In order to evaluate the selectivity of the ExBP-RT assays, we prepared a set of mixtures with the ratio of mutant/wildtype RNA ranging from 10 to 10^{-4} copies (10, 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) by mixing 10^7 copies of wildtype KRAS RNA with various amounts of mutant KRAS G12R RNA (from 10^8 copies to 10^3 copies). The mixtures were used to define the detection limit and range of the ExBP-RT assay for detection and quantification of the mutant KRAS G12R RNA by using quantitative PCR with SYBR Green I as the detection method (details in materials & methods section). Mutant KRAS G12R RNA was detected in clear distinction to the wildtype KRAS RNA in sample mixtures from 10 to 10^{-3} (10:1, 1:1, 1:10, 1:100, 1:1000) (**Figure 5-A**). Detection was linear from a mutant to wildtype ratio of 1:1000 to 10:1 (**Figure 5-B**, with r^2=0.9955 in least-squares analysis). The assay was verified using single RNA templates with wildtype or mutant KRAS G12R only (**Figure 6**). The PCR amplicons were confirmed by size (59 bp) on 2% agarose gel and visualized by SYBR Green staining (**Figure 7**).

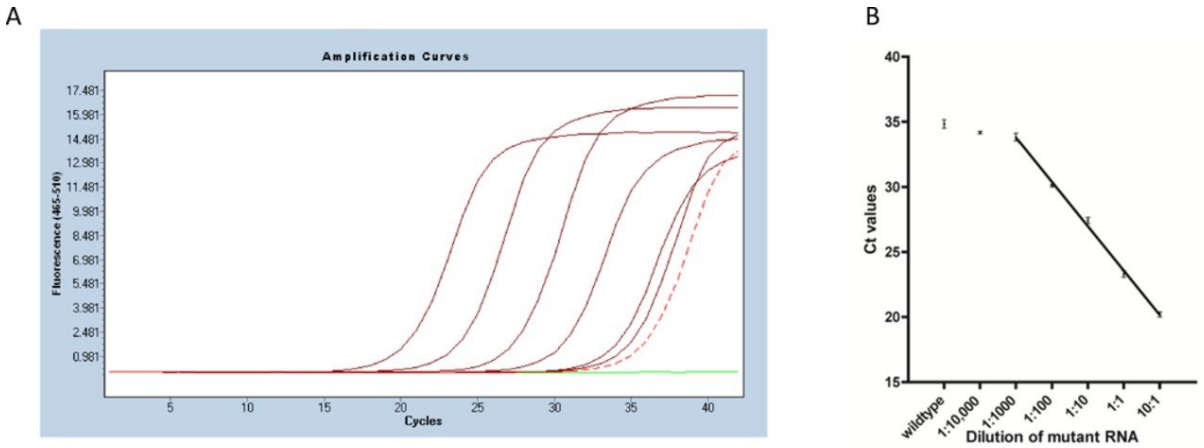


Figure 5: Detection of mutant KRAS G12R RNA with the ExBP-RT assay. (**A**) Representative qPCR amplification curves of mutant KRAS G12R RNA serially diluted into wild-type KRAS RNA (from left to right) 10:1, 1:1, 1:10, 1:100, 1:1000 and 1:10,000 (solid curves), wild-type KRAS RNA only (dash curve) and H_2O control (flat line). (**B**) The mean Ct values (three independent assays) were plotted against the dilution of mutant RNA. (Original article I, Ho, T. H., Dang, K. X., 2015) [122].

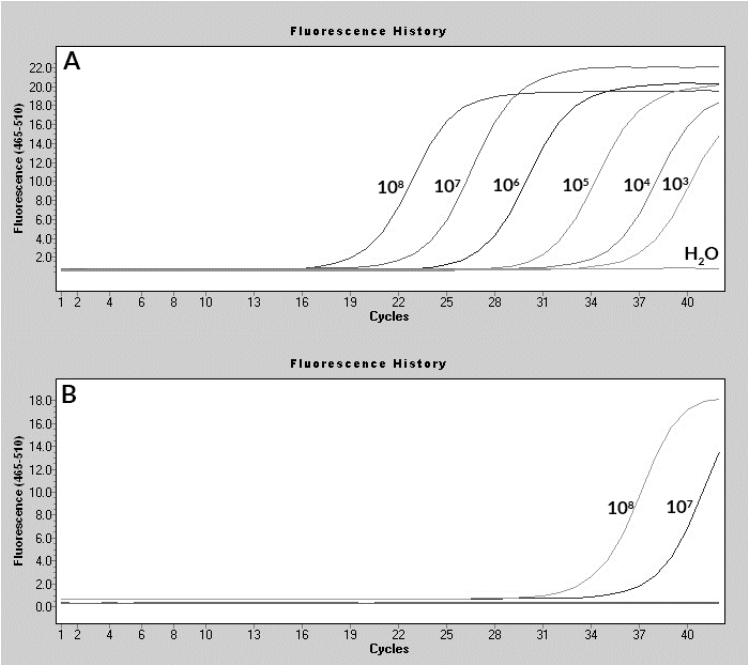


Figure 6: Representative amplification curves of ExBP-RT assay using single RNA templates. (A) qPCR amplification curves of mutant KRAS G12R RNA only (from left to right) 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, H_2O. (B) qPCR amplification curves of wildtype KRAS RNA only (from left to right) 10^8, 10^7. An extendable blocking probe was used in reverse transcription to suppress amplification of the wildtype KRAS RNA template. Due to cross-reactivity in reverse transcription, some amplification occurred in samples containing more than 10^7 copies of wildtype template. Samples containing 10^6 copies or less of wildtype KRAS RNA show no amplification. (Original article I, Ho, T. H., Dang, K. X., 2015) [122].

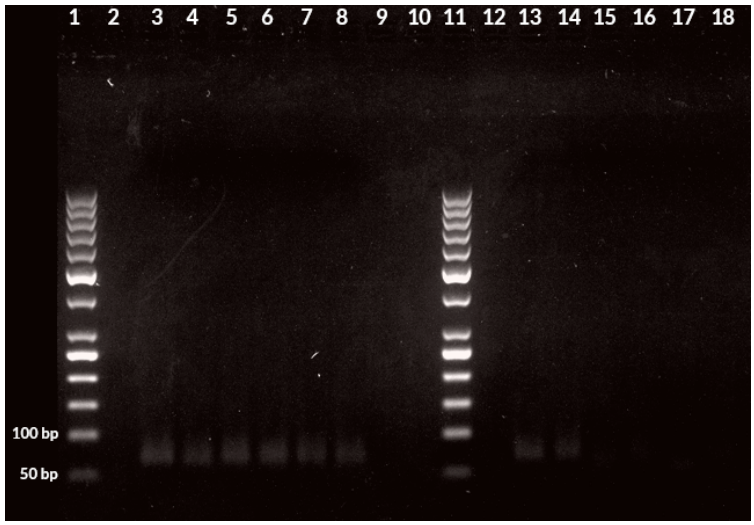


Figure 7: Visualization of ExBP-RT amplification products on agarose gel 2%. The expected size (59 bp) of specific amplified sequences has been confirmed after 42 cycles of PCR for all ExBP-RT assays containing 10^8, 10^7, 10^6, 10^5, 10^4, 10^3 copies of mutant KRAS G12R RNA (lanes 3-8, respectively) and those assays containing 10^8, 10^7 copies of wildtype KRAS RNA (lanes 13,14, respectively). No specific amplification product has been detected on gel for assays containing lower copy numbers of wildtype KRAS RNA (10^6, 10^5, 10^4, 10^3 copies, corresponding to lanes 15,16,17,18) or H_2O (lane 9). Lanes 1 and 11 are GeneRuler 50bp DNA ladder, ready-to-use (Thermo Scientific). Lanes 2,10 and 12 are blank wells. (Original article I, Ho, T. H., Dang, K. X., 2015) [122].

We also evaluated the selectivity of the ExBP-RT assays by analysing six different mutation types of the KRAS gene at codon 12 and the BRAF V600E mutation (the primer sequences are listed in **Table 3**). The selectivity of each ExBP-RT assay was determined by comparing the Ct values of a reaction containing a mismatched template with the Ct values of a second reaction containing matched templates at the same number of copies (details in materials & methods section). The selectivity was calculated as a percentage for each ExBP-RT assay ($2^{-\Delta Ct} \times 100\%$) (**Table 12)**. In summary, the ExBP-RT assays was able to detect different mutant KRAS and BRAF RNAs in the presence of a 1000 to 6,000-fold excess of wildtype transcripts at a selectivity ranging from 0.017% to 0.09%.

Table 12: The selectivity of ExBP-RT assays to detect different KRAS mutations at codon 12 and the BRAF V600E mutation. (Original article I, Ho, T. H., Dang, K. X., 2015) [122].

Mutations	ΔCt_{wt-mt}	Selectivity (%)
KRAS G12D (GGT>GAT)	11.4 ± 0.16	0.04 %
KRAS G12A (GGT>GCT)	10.5 ± 0.05	0.07 %
KRAS G12V (GGT>CTT)	11.3 ± 0.09	0.04 %
KRAS G12S (GGT>AGT)	10.2 ± 0.10	0.09 %
KRAS G12R (GGT>CGT)	12.5 ± 0.13	0.017 %
KRAS G12C (GGT>TGT)	12.2 ± 0.07	0.021 %
BRAF V600E (GTG>GAG)	12.5 ± 0.05	0.017 %

In order to initially validate the clinical applicability of the ExBP-RT assays, we utilized a probe-based quantitative PCR assay to detect the most common KRAS mutation type (G12D) (approximately 32.5% of all KRAS mutations) [126], using RNA from FFPE samples of colorectal cancer patients (the primer and probe sequences listed in **Table 3**). RNA was extracted from 11 FFPE CRC samples and diluted into 500 ng. The G12D mutation was clearly positive in one of 11 samples (**Figure 8-A**) with Ct value of 34.5 (right red curve). The positive control for reaction containing 10^7 copies of known mutant G12D RNA shows early amplification (left red curve). Whereas the negative controls with omission of RNA template or reverse transcriptase completely had no amplification. All clinical samples were verified to have a comparable amounts (0.6 – 1.8 x 10^5 copies/500 ng of total RNA) of total KRAS RNA (**Figure 9**), which is sufficient for the assay reproducibility [127]. The agarose gel electrophoresis of the positive sample and controls were confirmed to be of the expected size (59 bp) and with no specific band (no amplification) for the negative controls (**Figure 8-B**).

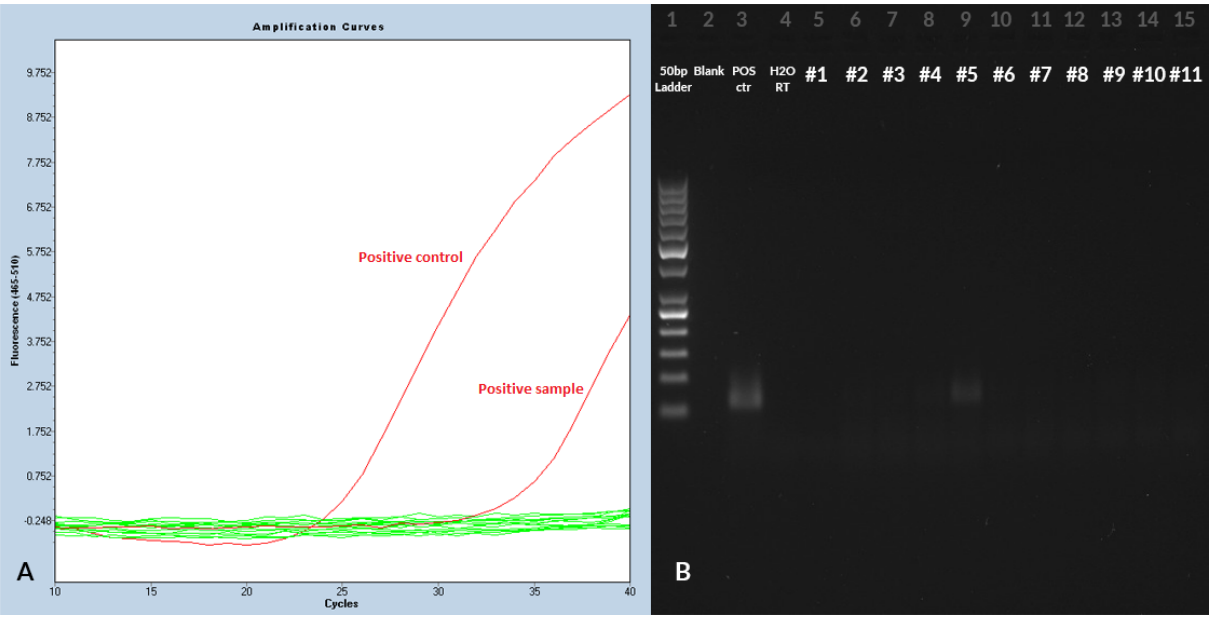


Figure 8: (**A**) Detection of mutant KRAS G12D RNA in FFPE samples from colorectal cancer patients. qPCR amplification curves of ExBP-RT assay for detection of mutant KRAS G12D RNA in 11 FFPE samples of colorectal cancer patients. (**B**) Visualization of ExBP-RT amplification products derived from FFPE clinical samples on agarose gel 2%. The expected size (59 bp) of specific amplified sequences has been confirmed for both the positive control (lane 3) and the positive- tested sample (lane 9 - sample #5). No specific amplification product has been detected on gel for all negative-tested samples (sample #1-4 and #6-11) as well as the negative control without template. Lanes 1 is GeneRuler 50bp DNA ladder, ready- to-use (Thermo Scientific). Lanes 2 is blank well. (Original article I, Ho, T. H., Dang, K. X., 2015) [122].

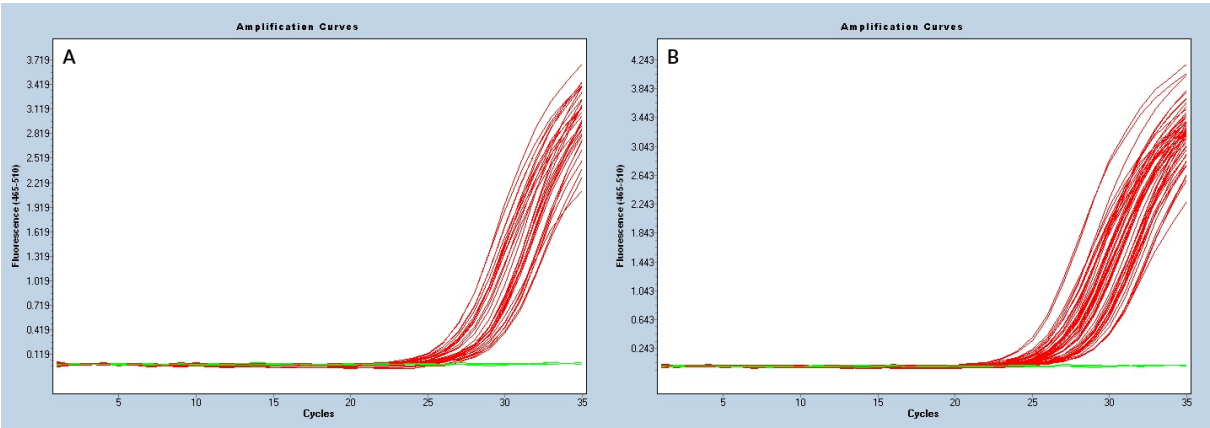


Figure 9: Quantification of total KRAS RNA from different clinical samples in multiplex ExBP-RT assay. Total amount of KRAS RNA from each clinical sample was quantified using a qPCR assay targeting a fragment upstream of the mutation site, which amplifies both the mutant and wildtype cDNA resulted from ExBP-RT assay. All KRAS mutation positive-tested samples (A) and negative-tested samples (B) showed comparable amount of total KRAS RNA. (Original article I, Ho, T. H., Dang, K. X., 2015) [122].

The multiplex ExBP-RT

Because the allele-specific step of the ExBP-RT assay is performed in reverse transcription during a single cycle, the problem of formation of primer-dimers in multiplex PCR assays is completely

avoided. Any redundant extendable blocking probes that remain unextended after reverse transcription and are transferred to the subsequent amplification reactions, are only 10 bp in length and cannot anneal to the templates under the relatively stringent PCR conditions. Designing multiplex assays based on ExBP-RT is thus, relatively straight forward and creates a possibility to simultaneously detect several mutations in a single tube. We designed a multiplex ExBP-RT assay to detect all six possible mutations within codon 12 of the KRAS gene, including the KRAS G12A, G12C, G12D, G12R, G12S and G12V mutations. The assay contains six different mutation-specific primers and a common extendable blocking probe targeting the wild-type KRAS transcript (sequences of primers and probe in **Table 3**). RNA (500 ng) extracted from 44 FFPE samples of colorectal cancer patients was used as templates. There was a clear amplification of the positive control (RNA extracted from cell-line A549) and no amplification of the wildtype control (RNA extracted from cell-line Colo205), or the negative controls (omission of RNA template or reverse transcriptase). We observed that 16 out of 44 samples (36.4%) were clearly positive for KRAS mutations at codon 12 (**Figure 10**). This mutation rate was slightly higher than that of previous studies using DNA-based techniques (27.7%) [83].

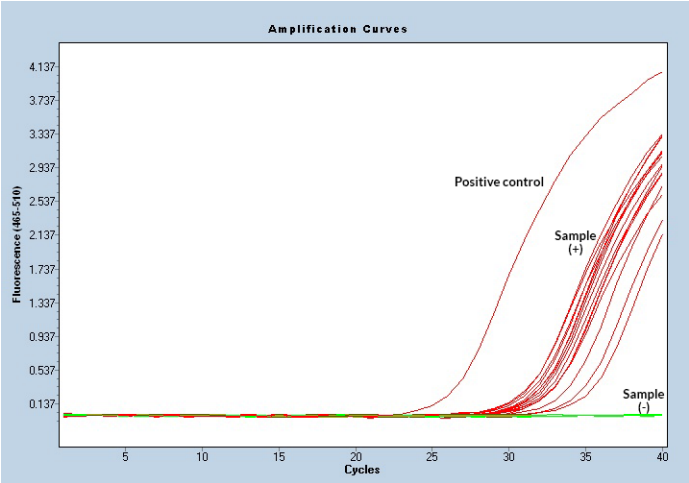


Figure 10: Simultaneous detection of all six possible KRAS mutations at codon 12 in FFPE samples from colorectal cancer patients. Representative qPCR amplification curves of multiplex ExBP-RT assay for detection of KRAS mutations at codon 12 in 44 FFPE samples of colorectal cancer patients. (Original article I, Ho, T. H., Dang, K. X., 2015) [122].

Comparison of ExBP-RT strategy to existing technologies

Reverse transcription with difference blocking probe options

In order to evaluate the efficacy of the competitive extendable blocking probe in the ExBP-RT assay, we conducted a comparison between reactions with an extendable blocking probe, with a non-extendable blocking probe or without a blocking probe. The BRAF V600E mutation detection assay was selected as model. The thermal program, reagents and mutant-specific primers were identical in all reactions. PCR amplification of the cDNA reverse transcribed from mutant BRAF V600E and corresponding and wildtype RNA template, without any blocking probe in the reverse

transcription reaction, resulted in a $\Delta Ct_{wt\text{-}mt}$ of only 4.4 (corresponding to selectivity of 5%) (**Figure 11-A**); the ExBP-RT assay with an extendable competitive blocking probe had a $\Delta Ct_{wt\text{-}mt}$ of 12.5 (selectivity of 0.017%) (**Figure 11-B**); and the assay with a non-extendable blocking probe had a $\Delta Ct_{wt\text{-}mt}$ of 5.8 (selectivity of 1.8%) (**Figure 11-C**). The selectivity of the non-extendable blocking probe assay was superior compared to the assay without any blocking probe, but it was 100 times lower than the selectivity of the ExBP-RT assay. These data clearly showed that an extendable wildtype-specific blocking probe outperformed corresponding non-extendable probes for suppression of mis-priming during reverse transcription.

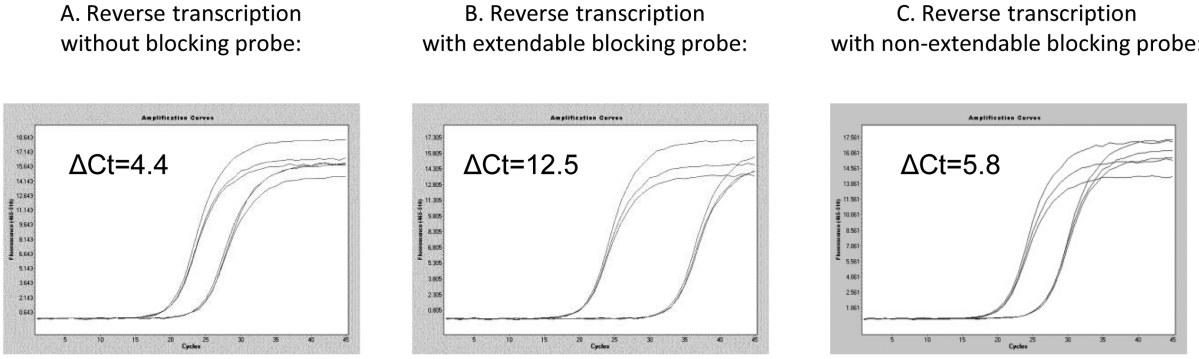


Figure 11: qPCR amplification curves (three replicates) derived from the same copy number of either mutant transcripts (left curves) or wildtype transcripts (right curves). cDNA synthesis reactions were performed either in the absence of competitive blocking probe (**A**), in the presence of an extendable competitive blocking probe (**B**) or in the presence of non-extendable competitive blocking probe (**C**). (Original article I, Ho, T. H., Dang, K. X., 2015) [122].

Selectivity of the ExBP assay compared to a similar DNA-based method

In order to evaluate the performance of the ExBP-RT assay, we designed a set of experiments to compare the selectivity of the ExBP-RT assay with other published DNA-based techniques.

The KRAS G12D (GGT>GAT) mutation was selected as model, because this RNA variant involves the most stable primer - template mismatch (dT:rG) [128]. A technique named allele-specific PCR with a blocking reagent (ASB-PCR) [129], was applied for this comparative experiment. This technique utilises a non-extendable blocking probe in the amplification step. In the ASB-PCR technique, discrimination between wildtype and mutant cDNA sequences is achieved by a mutant-specific primer that is shortened at its 5'-end to reduce its Tm to approximately 10°C below the annealing temperature and by addition of a blocking oligonucleotide, complementary to the wildtype sequence but phosphorylated at the 3'-end to prevent extension.

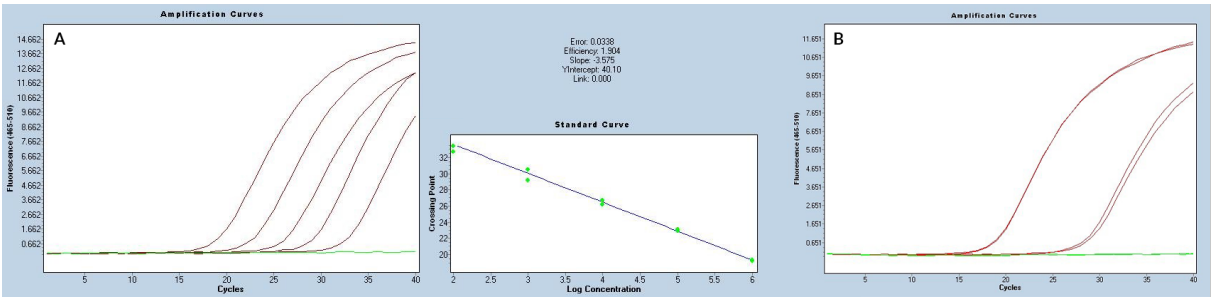


Figure 12: ASB-PCR assay (Mut2.1) for detection of mutant KRAS G12D RNA. (Original article I, Ho, T. H., Dang, K. X., 2015) [122].

A) Representative qPCR amplification curves of serial dilution and linear regression analysis showing a compromised PCR efficiency of only 90%.

B) qPCR amplification curves derived from the same copy number of either mutant transcript (left curves) or wildtype transcript (right curves).

The ExBP-RT assay for detection of mutant KRAS G12D RNA had a selectivity of 0.04%. The selectivity of corresponding ASB-PCR assay (originally named Mut2.1 assay), was only 0.15%. The $\Delta Ct_{wt\text{-}mt}$ value of this ASB-PCR assay was 10.1 (**Figure 12-B**), which translates into a relatively low selectivity, due to the low PCR amplification efficiency of only 90% (**Figure 12-A**). The PCR primer design with a low Tm and the inhibiting effect of blocking probe, probably compromise the PCR amplification efficiency of the ASB-PCR assay. When the blocking probe was excluded from the Mut2.1 assay, the selectivity of the assay was further decreased to about 20% ($2^{-2.3}$ x 100%). The Mut2.1 assay involves the primer - template mismatch (dA:dC), which is relatively unstable. When the discriminating primer was designed to target the same mutation but on the opposite strand (originally named Mut2.2 assay), to generate the most stable primer - template mismatch (dT:dG), the selectivity of the assay has been reported to be even further compromised [129].

2. THE PROGNOSTIC ROLE OF EXPRESSED KRAS AND BRAF MUTATIONS IN COLORECTAL CANCER

The expression of mutated KRAS and BRAF V600E mRNA in colorectal cancer

In order to analyse KRAS and BRAF mutations in colorectal cancer (CRC) we utilised the ExBP-RT assay to detect and quantify the RNA expression level of KRAS mutations at codon 12 and codon 13, as well as the expression level of the BRAF V600E mutation in a cohort of 571 CRC patients. We evaluated the prognostic role of the RNA expression of mutated KRAS, independently for each codon, and in relation to the expression of the BRAF V600E mutation. We found that the expression of mutated KRAS mRNA could be detected in the primary tumour tissue of 259 patients (45.4%) and expression of BRAF V600E mutation in 64 patients (11.2%). The detected KRAS mutations were at codon 12 in 36.1% and at codon 13 (G13D) in 10.7% of the cases. In 1,4% of the cases, we found KRAS mRNA transcripts mutated at both codons 12 and 13. KRAS mutations in mRNA occurred at a similar level in left-sided and right-sided disease (47.2% vs. 41.8%; p = 0.25). In contrast, RNA expression of the BRAF V600E mutation occurred

much more frequently in tumours located on the right side of the colon (20.4% vs. 6.3%; P < 0.001). A high level of mutated versus wildtype KRAS mRNA, defined as a $\Delta Ct_{mt\text{-}wt}$ value of less than 10, was observed in 45.6% of patients with KRAS mutations at codon 12 (94/206) and in 34.4% of patients with the KRAS G13D mutation. A high level of mutated KRAS mRNA was detected at a similar frequency in right-sided and left-sided CRC (27.5% vs 20.2%).

Disease-specific survival and the prognostic value of mutated KRAS mRNA

After a median follow-up of 5.9 years, the 5-year disease-specific survival (DSS) rate of the study population was 62.2% (95% CI 58.0–66.3%). The DSS among patients with mRNA expression of the BRAF V600E mutation was significantly worse compared to those expressing only wild-type BRAF [5-year DSS rate: 42.3% (95% CI 29.7–54.9%) versus 64.7% (95% CI 60.4–69.1%); log-rank P < 0.001; HR 2.10 (95% CI 1.47–3.00; P < 0.001)]. Both KRAS and BRAF V600E mutations were present in 12 patients (2.1%). Only one of these patients had a high level of mutated KRAS mRNA (0.18%) and he/she died only 3.8 months after diagnosis. We considered the BRAF V600E mutation to be a significant molecular confounder in the survival analysis of patients with CRC, since it has been previously established as marker of poor prognosis in CRC [88, 130]. In order to determine the prognostic value of expressed KRAS mutations, independently to the BRAF V600E mutation, we excluded all patients with tissue expression of the BRAF V600E mutation from further analysis. Among the remaining patients, expression of mutated KRAS mRNA in the primary tumour tissue, was associated with a worse prognosis than expression of only wild-type KRAS [5-year DSS: 58.1% (95% CI 51.7–64.6%) vs. 71.1% (95% CI 65.3–76.8%); log-rank p = 0.005; HR 1.49 (95% CI 1.12–1.98); p = 0.006; **Figure 13**].

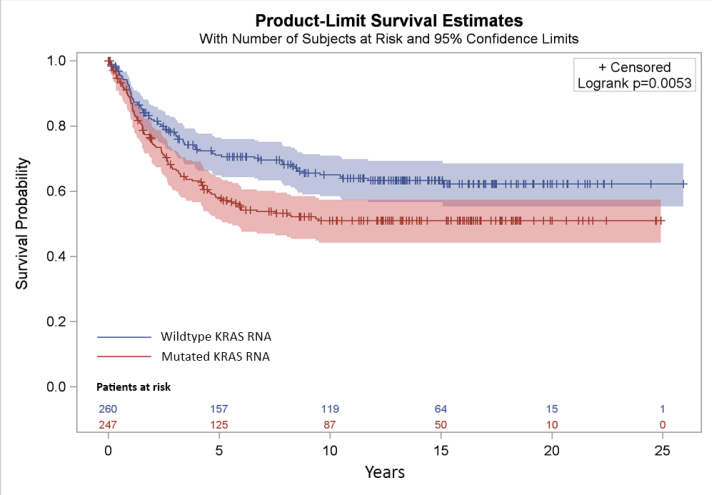


Figure 13: Prognostic impact of mutated KRAS mRNA expression on disease-specific survival (DSS) of wild-type BRAF colorectal cancer (CRC) patients. Kaplan–Meier survival curves show DSS for CRC patients (n = 507) expressing wild-type vs. mutated KRAS mRNA in the primary tumour tissue. Only patients expressing wild-type BRAF were included in the analysis; thus, 64 patients expressing the $BRAF^{V600E}$ mutation were excluded (Original article IV, Dang, K. X., Ho, T. H., 2021 - Unpublished).

Side-specific and prognostic impact of mutated KRAS mRNA

In order to explore the association between expressed KRAS mutations and prognosis, we compared the survival of patients with high, low or no expression of mutated KRAS mRNA, among patients with left- versus right-sided CRC. We found a significant difference in survival between patients with different levels of mutant KRAS expression in the primary tumours among patients with left-sided disease. The difference in DSS between patients with high, low and no tumour expression of mutated KRAS mRNA was highly significant [5-year DSS rate: 40.8% (95% CI 28.7–52.9%), 61.3% (95% CI 51.2–71.4%) and 69.9% (95% CI 62.9–76.9%); log-rank P < 0.001; HR for high versus no expression 2.27 (95% CI 1.53–3.36), P < 0.001; and HR for low versus no expression 1.30 (95% CI 0.87–1.93), p = 0.200; **Figure 14-B**]. However, among those with right-sided tumours the prognosis was not statistically different between patients with high, low and no tumour expression of mutated KRAS mRNA [5-year DSS rate: 65.5% (95% CI 50.8–80.2%), 73.0% (95% CI 57.9–88.1%) and 73.6% (95% CI 63.4–83.8%); log-rank p = 0.726; HR for high versus no expression 1.29 (95% CI 0.68–2.41), p = 0.432; and HR for low versus no expression 1.06 (95% CI 0.52–2.18), p = 0.868; **Figure 14-A**].

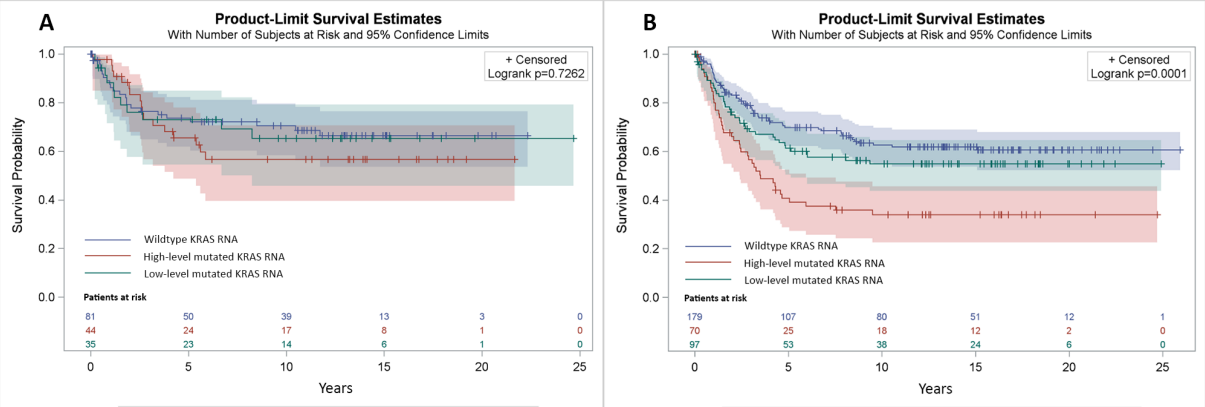


Figure 14: Prognostic impact of mRNA expression of KRAS mutations on disease-specific survival (DSS) in right- vs. left-sided colorectal cancer (CRC). Kaplan-Meier survival curves displaying DSS in CRC patients (n = 507) with high, low and no expression of mutated KRAS mRNA in the primary tumour tissue. Only patients expressing wild-type BRAF were included in the analysis; thus, 64 patients expressing the $BRAF^{V600E}$ mutation were excluded. No statistically significant differences between the groups were detected in right-sided disease (**A**). In contrast, the difference in DSS between patients with a high, low and no expression of mutated KRAS mRNA was highly significant among those with left-sided tumours [5-year DSS rate: 40.8% (95% CI 28.7–52.9%), 61.3% (95% CI 51.2–71.4%) and 69.9% (95% CI 62.9–76.9%); logrank P < 0.001; HR for high expression versus no expression 2.27 (95% CI 1.53–3.36), P < 0.001] (**B**) (Original article IV, Dang, K. X., Ho, T. H., 2021 - Unpublished).

Stratification according to TNM stage

Among 346 CRC patients with left-sided tumours and without expression of the BRAF V600E mutation, a high level of mutated KRAS mRNA was more frequently detected in patients with stage IV disease in comparison with non-metastatic stages I, II and III (31,6% vs 11.8%, 19.4% and 16.7%, respectively, p = 0.0036). Stratification analysis according to TNM stage for this group of patients, revealed a poor prognosis in patients with a high expression of KRAS mutations

specifically in stage III disease, with 5-year DSS rates for a high, low and no expression of mutated KRAS mRNA of 28.6% (95% CI 4.9–52.2%), 52.9% (95% CI 33.4–72.2%) and 70.2% (95% CI 56.6–83.8%); log-rank p = 0.001; HR for high versus no expression 3.89 (95% CI 1.87–8.08), p = 0.0003 and HR for low versus no expression 1.83 (95% CI 0.9–3.71), p = 0.096 (**Figure 15-III**). In a subsequent multivariate analysis, adjusting for age, gender and rectum or colon location, we were able to confirm that the negative prognostic impact of the mutated KRAS mRNA expression was limited to left-sided stage III CRC [HR for high versus no expression 3.18 (95% CI 1.48–6.87), p = 0.0032 and HR for low versus no expression 1.91 (95% CI 0.93–3.94), p = 0.0788]. Most deaths in this cohort were secondary to metastatic recurrence and the level of mutated KRAS mRNA in the primary tumour tissue thus, indirectly correlated with the metastatic potential of the disease.

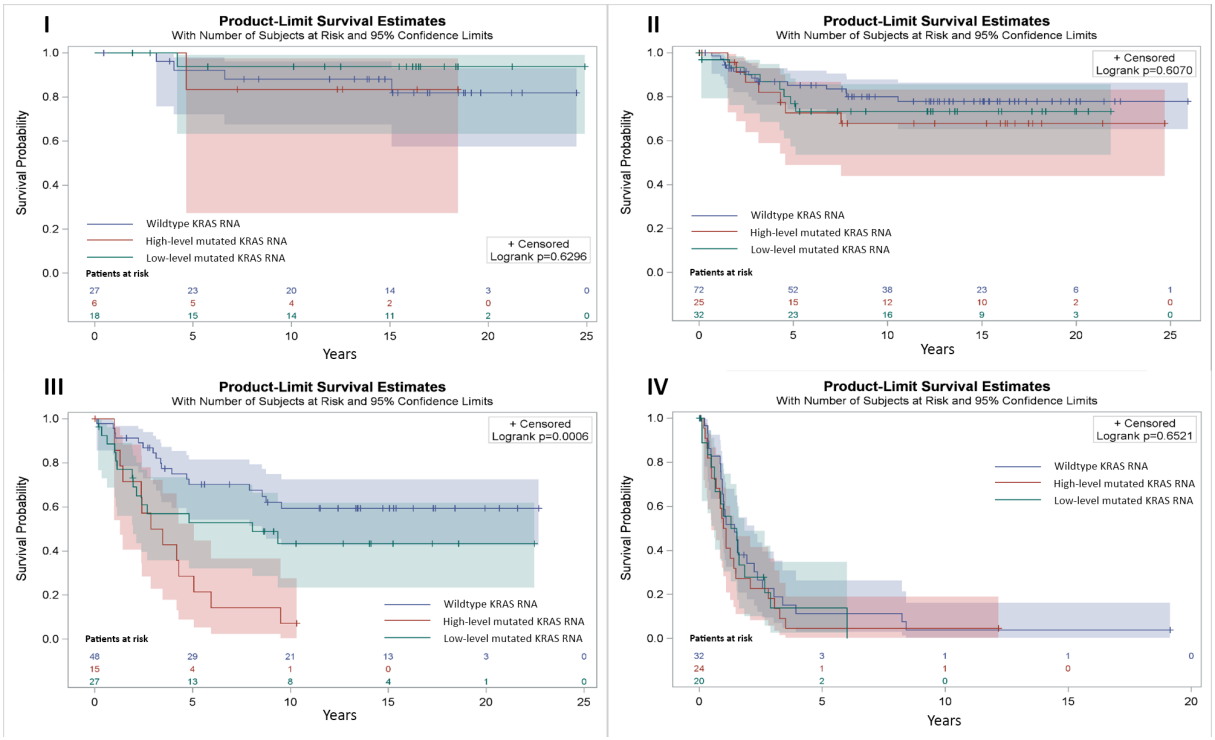


Figure 15: Prognostic impact of mRNA expression of *KRAS* mutations on disease-specific survival (DSS) in left-sided colorectal cancer (CRC) stratified by TNM stage. Kaplan–Meier survival curves displaying DSS among left-sided CRC patients (n = 346) with a high, low and no expression of mutated *KRAS* mRNA in the primary tumour tissue. Only patients expressing wild-type *BRAF* were included in the analysis. Stratification according to stage revealed a distinct negative prognostic impact of mutated *KRAS* mRNA expression in the primary tumour tissue of locally advanced stage III patients, with 5-year DSS rates for high, low and no expression of mutated *KRAS* mRNA of 28.6% (95% CI 4.9–52.2%); 52.9% (95% CI 33.4–72.2%) and 70.2% (95% CI 56.6–83.8%; logrank p = 0.001); HR for high vs. no expression 3.89 (95% CI 1.87-–8.08; p = 0.0003) (Original article IV, Dang, K. X., Ho, T. H., 2021 - Unpublished).

3. Expressed BRAF V600E mutations in thyroid cancer

Sensitivity of the BRAF V600E mRNA mutation detection assay

At first, we evaluated the sensitivity of the ExBP-RT-based mRNA mutation detection assay for BRAF V600E, using in vitro transcribed mutant and wildtype mRNA as templates (**Figure 16**). The same amount of mutant and wildtype RNA templates (10^7 copies) were used in the qRT-PCR reactions. We observed that the mutant BRAF V600E mRNA was amplified 14.67 cycles earlier than the wildtype BRAF mRNA. The mis-priming of the mutation-specific primer to the wildtype BRAF mRNA template was calculated as a percentage by the equation $2^{-\Delta Ct} \times 100\%$, where ΔCt is the difference in amplification threshold values of qRT-PCR amplification. The relative mis-priming efficiency was approximately 0.005% of the specific priming efficiency ($2^{-14.67} \times 100\%$). As a result, the mutation detection assay could detect the BRAF V600E mutation in mRNA in the presence of a 10000-fold excess of wildtype BRAF mRNA.

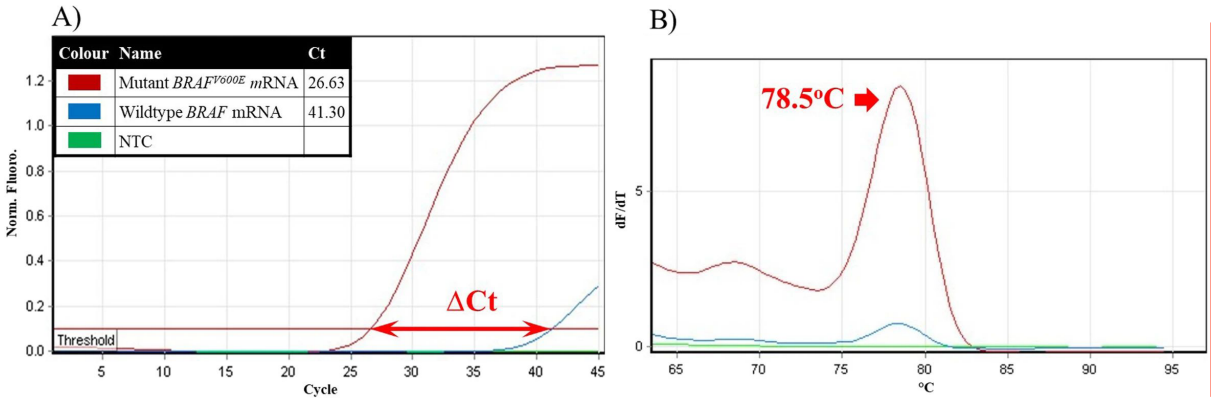


Figure 16: Detection sensitivity for BRAF V600E mutation in mRNA. The sensitivity of a novel mRNA-based mutation assay for BRAF V600E was determined using 10^7 copies of in vitro transcribed mRNA containing the BRAF V600E mutation and the same amount of corresponding wildtype mRNA as templates. (Original article III, Dang, K. X., Tran, T. V., 2020) [124]

A) Amplification signal from mutant BRAF V600E mRNA (red line), wildtype BRAF mRNA (blue line) and no-template control-NTC (green line)

B) Corresponding melting peaks of the amplification products

Detection of the BRAF V600E mutation in mRNA and DNA from benign and malignant thyroid FFPE tissue samples

The mRNA-based mutation detection assay for BRAF V600E was used to analyse the FFPE tissue samples of thyroid tumours and non-malignant thyroid disease and the results were compared with Sanger sequencing (**Figure 17**). We detected mutant BRAF V600E mRNA in 18 of 32 thyroid cancer samples (56.3%). In comparison, mutant BRAF V600E DNA was detected by sequencing in only 13 of 32 thyroid cancer samples (40.6%), all of which were among the 18 samples positive for BRAF V600E mRNA (**Figure 18**). There was a statistically significant difference between the sensitivity of the two tests in detection of the BRAF V600E mutation ($p = 0.0736$, McNemar's two-tailed chi-square test). No BRAF V600E mutations were detected in any of the 30 FFPE samples of benign thyroid tissues by either technique, indicating a high specificity of both assays.

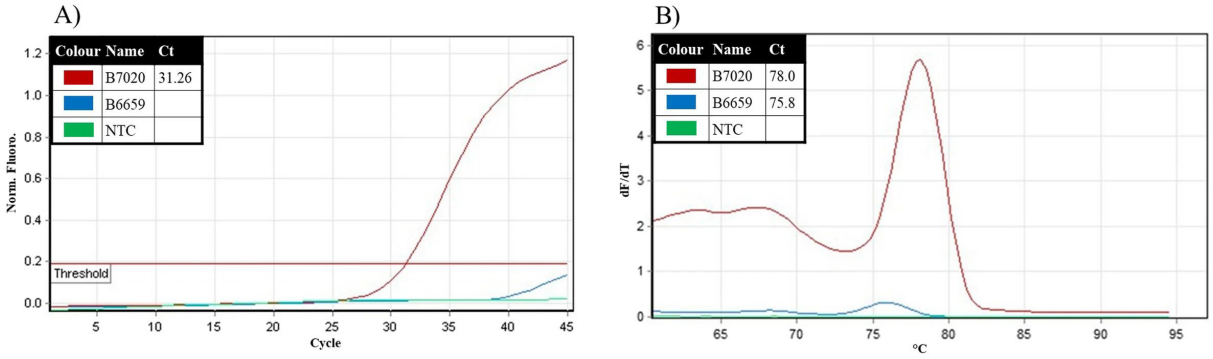


Figure 17: Detection of BRAF V600E mutation in mRNA from clinical FFPE samples. BRAF V600E mRNA-based mutation assay was utilized for ultrasensitive detection of the BRAF V600E mutation in mRNA isolated from clinical FFPE specimens of thyroid cancer and non-malignant thyroid disease. (Original article III, Dang, K. X., Tran, T. V., 2020) [124]

A) Amplification signals from a sample containing mutant BRAF V600E mRNA (B7020 - red line), a sample without mutant BRAF V600E mRNA (B6659 - blue line) and no-template control (NTC - green line)

B) Corresponding melting peaks of the amplification products

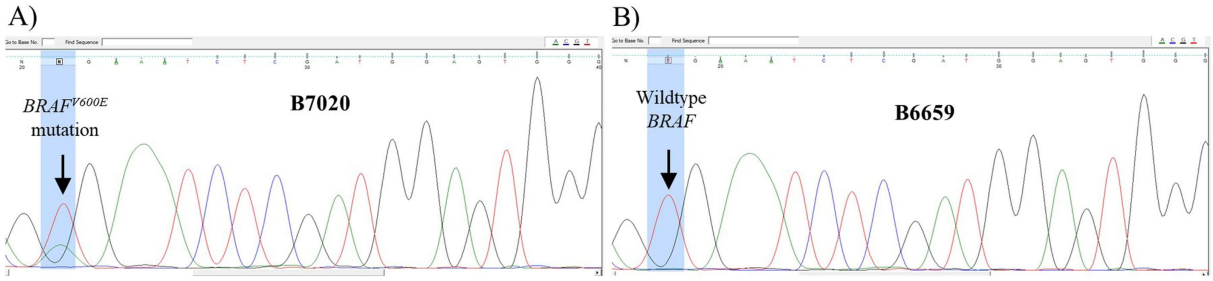


Figure 18: Detection of the BRAF V600E mutation in FFPE samples using DNA sequencing. Sanger DNA sequencing was used as a reference method to detect the BRAF V600E mutation in clinical FFPE specimens from patients with thyroid cancer and non-malignant thyroid disease. (Original article III, Dang, K. X., Tran, T. V., 2020) [124]

Sequencing chromatogram shows:

A) Two peaks (red and green) at the nucleotide position of interest for a sample with the BRAF V600E mutation (B7020)

B) Single peak (red) for a sample with wildtype BRAF only (B6659)

Determination of relative expression levels of the BRAF V600E mRNA versus wildtype BRAF mRNA

We further analysed the allele-specific expression of the mutant and wildtype alleles of the BRAF gene in the 13 thyroid cancer samples, where BRAF V600E mutants had been detected in both DNA and mRNA (**Table 13**). The relative abundance of the BRAF V600E mutation versus wildtype BRAF in DNA ranged between 0.170–0.703. On the mRNA level, we observed a much greater variation in the relative level of the respective alleles with a range of about 3 logs (0.001–0.429). This finding could indicate that the expression level of the BRAF V600E gene can be highly variable in thyroid cancer and maybe in other cancers as well.

Table 13: Clinicopathologic and molecular data of novel mRNA-based assay and Sanger sequencing for BRAF V600E expression of thyroid cancer cases. (Original article III, Dang, K. X., Tran, T. V., 2020) [124]

Case No.	Sex	Tumor type	Molecular data				
			Novel mRNA-based assay[b]		Sequencing[c]		Log (R^{RNA}/R^{DNA})
			ΔCt (mt-wt)	R^{RNA}	nt 1799	R^{DNA}	
1	1	Malignant	2.58	0.16724	1799T>A	0.703	-0.623
2	1	Malignant	5.71	0.0191	1799T>A	0.6	-1.497
3	1	Malignant	4.97	0.03191	1799T>A	0.48	-1.177
4	1	Malignant	1.22	0.42928	1799T>A	0.378	0.056
5	1	Malignant	1.81	0.28519	1799T>A	0.368	-0.11
6	1	Malignant	2.84	0.13966	1799T>A	0.313	-0.35
7	2	Malignant	10	0.00098	1799T>A	0.295	-2.48
8	1	Malignant	2.15	0.22531	1799T>A	0.28	-0.094
9	1	Malignant	4.87	0.0342	1799T>A	0.265	-0.889
10	1	Malignant	4.35	0.04904	1799T>A	0.228	-0.666
11	1	Malignant	1.41	0.37631	1799T>A	0.208	0.259
12	1	Malignant	6.56	0.0106	1799T>A	0.178	-1.224
13	1	Malignant	3	0.125	1799T>A	0.17	-0.134
14	2	Malignant	2.38	0.19211	1799T	0	n.a.
15	1	Malignant	4.08	0.05913	1799T	0	n.a.
16	1	Malignant	6.52	0.0109	1799T	0	n.a.
17	1	Malignant	7.62	0.00508	1799T	0	n.a.
18	1	Malignant	8.15	0.00352	1799T	0	n.a.
19	1	Malignant	wt	0	1799T	0	n.a.
20	1	Malignant	wt	0	1799T	0	n.a.
21	1	Malignant	wt	0	1799T	0	n.a.
22	1	Malignant	wt	0	1799T	0	n.a.
23	1	Malignant	wt	0	1799T	0	n.a.
24	1	Malignant	wt	0	1799T	0	n.a.
25	1	Malignant	wt	0	1799T	0	n.a.
26	1	Malignant	wt	0	1799T	0	n.a.
27	1	Malignant	wt	0	1799T	0	n.a.
28	1	Malignant	wt	0	1799T	0	n.a.
29	1	Malignant	wt	0	1799T	0	n.a.
30	1	Malignant	wt	0	1799T	0	n.a.
31	1	Malignant	wt	0	1799T	0	n.a.
32	1	Malignant	wt	0	1799T	0	n.a.
33	1	Benign	wt	0	1799T	0	n.a.
34	1	Benign	wt	0	1799T	0	n.a.
35	2	Benign	wt	0	1799T	0	n.a.
36	1	Benign	wt	0	1799T	0	n.a.
37	1	Benign	wt	0	1799T	0	n.a.
38	2	Benign	wt	0	1799T	0	n.a.
39	1	Benign	wt	0	1799T	0	n.a.
40	1	Benign	wt	0	1799T	0	n.a.
41	1	Benign	wt	0	1799T	0	n.a.

42	1	Benign	wt	0	1799T	0	n.a.
43	2	Benign	wt	0	1799T	0	n.a.
44	2	Benign	wt	0	1799T	0	n.a.
45	1	Benign	wt	0	1799T	0	n.a.
46	1	Benign	wt	0	1799T	0	n.a.
47	1	Benign	wt	0	1799T	0	n.a.
48	1	Benign	wt	0	1799T	0	n.a.
49	1	Benign	wt	0	1799T	0	n.a.
50	1	Benign	wt	0	1799T	0	n.a.
51	1	Benign	wt	0	1799T	0	n.a.
52	1	Benign	wt	0	1799T	0	n.a.
53	1	Benign	wt	0	1799T	0	n.a.
54	1	Benign	wt	0	1799T	0	n.a.
55	1	Benign	wt	0	1799T	0	n.a.
56	1	Benign	wt	0	1799T	0	n.a.
57	1	Benign	wt	0	1799T	0	n.a.
58	1	Benign	wt	0	1799T	0	n.a.
59	1	Benign	wt	0	1799T	0	n.a.
60	2	Benign	wt	0	1799T	0	n.a.
61	1	Benign	wt	0	1799T	0	n.a.
62	1	Benign	wt	0	1799T	0	n.a.

[a] All cases were diagnosed with differentiated thyroid cancer.

[b] Novel mRNA-based assay was used to calculate the relative abundance of mutant versus wildtype allele at the mRNA level using the delta Ct value (ΔCt) between the mutant and wildtype signals: $R^{RNA} = 1/2^{\Delta Ct(BRAF\_V600E - BRAF\_wildtype)}$.

[c] Sanger sequencing was used to calculate the relative abundance of mutant versus wildtype allele at the DNA level using the peak heights (H) at the nucleotide position of interest (1799T>A) on a DS chromatogram: $R^{DNA} = H^{BRAF\_V600E} / H^{BRAF\_wildtype}$.

wt: wildtype; **mt**: mutant; **n.a**: no applicable.

4. Expression of KRAS and BRAF mutations in esophageal atresia

Sample cohort and RNA viability

We retrospectively examined 112 tissue biopsies, taken at gastro-duodenoscopy from 61 adult patients under follow-up after surgical treatment for esophageal atresia during infancy. mRNA extracted from the samples was converted to cDNA using the ExBP-RT assay and cDNA products were then analysed by real-time PCR. The viability of the mRNA in the samples was confirmed by analysing the mRNA expression of wildtype KRAS and BRAF in the biopsy samples.

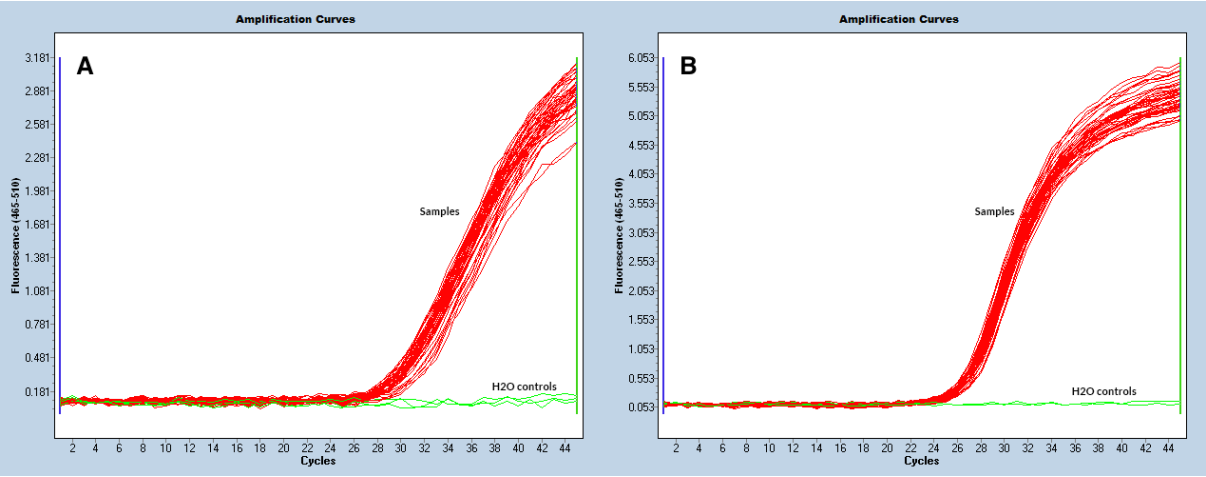


Figure 19: (**A**) Total KRAS detection in qPCR. (**B**) Total BRAF detection in qPCR. qPCR, quantitative polymerase chain reaction. (Original article II, Dang, K. X., 2018) [125]

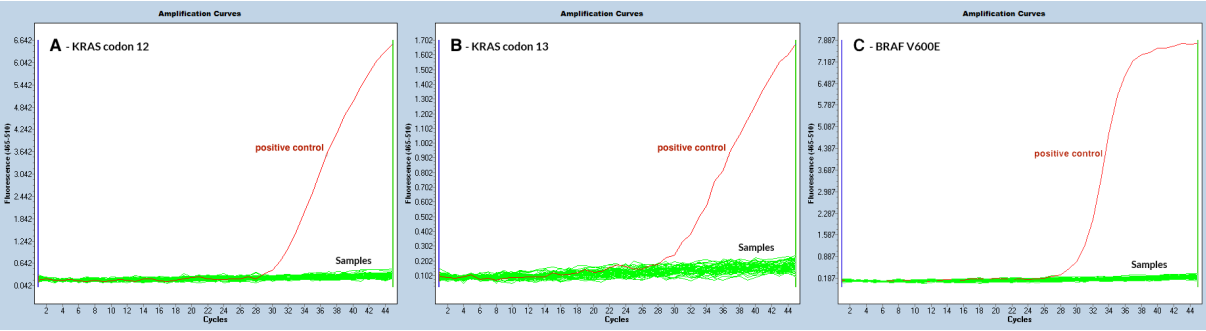


Figure 20: (**A**) KRAS codon 12 mutation detections in qPCR. (**B**) KRAS codon 13 mutation detections in qPCR. (C) BRAF mutation detection in qPCR. qPCR, quantitative polymerase chain reaction. (Original article II, Dang, K. X., 2018) [125]

The tissue expression levels of total KRAS and BRAF mRNA was analysed by real-time PCR with primer pairs that co-amplified both mutant and wildtype cDNAs. Expression of wildtype KRAS and BRAF RNA was readily detected in all 112 EA samples, as well as in positive controls. The expression level of wildtype KRAS and BRAF mRNA was similar in all samples (**Figure 19**). No amplification was detected in the negative, H_2O controls.

Detection of expressed KRAS and BRAF mutations

Positive control samples included mutation controls, consisting of RNA extracted from cell lines that expresses a KRAS mutation at codon 12 (A549), another cell line that expresses a KRAS mutation at codon 13 (Lovo) and finally a cell line that expresses the BRAF V600E mutation (Colo205) (**Figure 20**). Negative controls (wild-type cell line) and non-sample control (PCR water) were included in every experiment. Although wildtype KRAS and BRAF RNA was consistently detected in all samples, expression of KRAS codon 12, codon 13, or BRAF mutations could not be detected in any of the 112 patient samples.

DISCUSSION

ExBP-RT

The role of allele discrimination

DNA-based strategies for SNP genotyping and mutation detection consist of a sequence of steps, in which, the target DNA amplification is usually performed as first [131], then followed by allele discrimination and detection of allele-specific products [132]. There are several limitations associated with this approach. When the targeted DNAs are amplified prior to an allele discrimination reaction, both mutant and wildtype variants are amplified with the same efficiency. The wildtype variant is usually much more abundant than the mutant variant and competes with the mutant variant during the amplification. Subsequently, the mutated DNA product resulting from a low abundance variant, may not be amplified sufficiently to surmount the lower detection limit of the assay. Also, the discriminating power of allele-specific primers will be compromised when the target amplification step coincides with the allele discrimination reaction, due to some of the characteristics, i.e. annealing at relatively high reaction temperatures and design restriction to avoid primer dimers, that are required for primers in an amplification reaction such as allele-specific PCR [132, 133]. In addition, the techniques which require post-amplification handling of products, will increase the risks of laboratory contamination [134-136].

In the ExBP-RT strategy that was developed and utilised in this thesis project, the allele discrimination reaction is performed in the first step, taking place during reverse transcription. Therefore, the decay of allele discriminating power, due to non-specific cross-priming during amplification, will be avoided. Most importantly, since the reverse transcription reaction is performed in a single cycle and the reaction time can be longer than the typical annealing, extension times during PCR, primers can optimize solely for the purpose of allele discrimination, without interference from concurrent optimization for PCR performance. In practice, the length of the mutation-specific primers and blocking probes used in ExBP-RT have been designed to be significantly shorter than typical PCR primers, with a length of only about 10 nucleotides (Tm 37-50°C). The shorter primers have a superior capacity for discrimination between template variants that differ at only a single nucleotide position, since any mismatch generated during a non-specific priming event causes a relatively greater decrease in binding affinity, as compared to a PCR primer, typically at least 16 nucleotides in length.

Several improvements to previously existing amplification technologies have been presented by other authors, most recently by using artificial mismatched nucleotides on allele-specific primers to improve discrimination between the template variants in the sample and externally added control sequences [137]. Many other sensitive mutation detection assays based on the principle of allele-specific PCR have also been described [138-140]. All of these technologies are, however, to some degree hampered by non-specific cross priming during amplification, leading to a decay in the discriminating power over each cycle of amplification [132, 133]. The rate of non-specific cross-priming is dependent on which specific nucleotide has been used for discrimination between the variant template sequences and PCR product yields have been shown to decrease by 20-fold for A:A mismatches, whereas mismatches involving thymine (T) have minimal effect on PCR product

yield [141]. Therefore, the design of AS-PCR assays for detection of the T > A mutation (e.g. BRAF V600E - 1799 T > A), which involves A:A or T:T mismatches, is inherently challenging, restricting assay sensitivity to about 0.1% at best [142-148]. The ExBP-RT technique discriminates between wildtype and mutant alleles during a single cycle of reverse transcription, completely eliminating the problem of decay of sensitivity during subsequent qPCR amplification [122].

The difference of non-extendable vs. extendable wildtype-specific blocking probes

In competitive allele-specific PCR assays, non-extendable wildtype-specific blocking probes have been widely used to reduce mis-priming and thus, to improve the selectivity of reaction [129, 149-157]. The blocking probes suppress mis-priming of the mutation-specific primers by blocking the potential priming site upon hybridization to the wildtype template.

In the first paper of this thesis, we have shown that the extendable wildtype-specific blocking probes exhibit superior performance on suppression of mis-priming during reverse transcription in comparison with non-extendable wildtype-specific blocking probes. The underlying mechanism of this difference comes from the extension of the extendable wildtype-specific blocking probes upon hybridization of ExBP to the wildtype template, extension to generate a wildtype cDNA that forms a wildtype cDNA-RNA hybrid. The extension reaction is much faster than annealing, and the formed cDNA-RNA hybrid is much more thermostable than hybridization of any non-extendable oligonucleotide probe due to the significantly greater length. Therefore, it efficiently blocks the priming sites on the wildtype RNA templates from mis-priming by the mutation-specific primers, when the reaction temperature is gradually decreased towards the optimal annealing temperature. This ultimately results in effective avoidance of mis-priming and dramatically improves the selectivity of the ExBP-RT assay.

The challenge of PCR optimization

Unlike allele-specific PCR, usually requiring stringent optimization for PCR reaction conditions [138, 139, 158-165], the ExBP-RT assays are relatively simple to design and set up, with only minimal optimization required. Apart from the extendable blocking probes, all major components of the reverse transcription reaction are included in typical commercially available kits and the components are added according to the manufacturers' instructions. We utilised a universal protocol for detection of all mutation types on different genes. Furthermore, the performance of the ExBP-RT assays seems less likely to be less influenced by specific differences in the nucleotide sequence of the wildtype and mutated RNA variants, in comparison to AS-PCR [132]. Our results show that the differences in sensitivity, between different ExBP-RT assays varied within a relatively narrow range (10^{-3} - 10^{-4}). Even in the ExBP-RT assays for KRAS G12D (GGT>GAT) and KRAS G12S (GGT>AGT) mutations, which involve the most stable primer-template mismatch (dT:rG) [128], we were able to achieve a selectivity of $4x10^{-4}$ and $9x10^{-4}$, respectively. A critical feature of the ExBP-RT assay is that low abundance mutant alleles can be enriched by selective PCR amplification, based on an engineered priming sequence inserted into the 5'-tail of the mutant cDNA during reverse transcription. [166] Thus, only mutant the cDNA product comprising the engineered 5'-tail sequence is amplified during PCR. The ExBP-RT technique can be generally applied to detect practically any RNA variant in the presence of an excessive background of

wildtype counterparts and also the alternative variants regardless of their relative abundance. As an example, we have performed ExBP-RT assays for detection of mutant KRAS G12D RNA variant in an excessive background of another mutant variant, KRAS G12A RNA, the assay displaying an excellent selectivity of 0.05% with a $\Delta Ct_{wt\text{-}mt}$ of 11.01 cycles (**Figure 12**).

Analysis of expressed KRAS and BRAF mutations in CRC

The ExBP-RT assays were used to detect the level of mutated KRAS and BRAF mRNA in primary CRC tumour tissue. After analysing the association between the survival and the level of mutated KRAS mRNA, we found that patients with a high level of mutated KRAS mRNA in a left-sided, locally advanced disease, had a strikingly worse prognosis than corresponding patients with expression of wildtype KRAS mRNA only. Surprisingly, this association was completely absent in right-sided disease. Our results are consistent with other reports, describing the different behaviours and biology of right-sided and left-sided CRC [167]. Some reports indicate that the discrepancy might reflect a difference in the tumour's dependence on KRAS-associated signalling [168-172]. Other possible determinant factors include the EGFR ligands - epiregulin (EREG) and amphiregulin (AREG), that recently have been reported to be expressed at significantly higher levels in left-sided CRC. Involvement of such EGFR ligands could stimulate KRAS-related pathways [173-175]. Interestingly, studies performed in the eighties, based on semi quantitative immunohistochemistry using the RAP-5 monoclonal antibody, suggested a correlation between the expression of the Ras oncogene p21 protein and the metastatic potential and prognosis in rectal cancer [92, 93, 176, 177]. These findings could support our finding regarding the expression of mutant KRAS in left-sided CRC, although there has been some dispute regarding the specificity of the RAP-5 monoclonal antibody.

The strong prognostic impact of mutated KRAS mRNA that we observed in left-sided CRC was most evident in local advanced stage III, BRAF V600E-negative disease. The 5-year DSS of patients with a left-sided stage III CRC and a high level of mutated KRAS mRNA in the primary tumour was 28.6%, corresponding to a similar risk of recurrence and death as patients with a stage IV disease (**Figure 15-Stage III, IV**). On the contrary, among the patients negative for BRAF V600E in our cohort, the 5-year DSS of stage III and left-sided CRC patients with no mutated KRAS mRNA in the primary tumour tissue was 70.2%, similar to the survival of patients with localized stage II disease (**Figure 15-Stage II, III**). This suggests that the expression level of the of mutated KRAS mRNA in the primary tumour, could be a useful biomarker for stratification of patients with left-sided stage III CRC into high- and low-risk groups. The capacity to stratify patients with a locally advanced stage III disease could be highly useful for individualizing treatment regimens and the duration of adjuvant chemotherapy and possibly as an inclusion criterion clinical trials on novel therapeutic agents.

There is an imminent need for prognostic indicators that could help identifying patients most likely to benefit from an intensified recurrence surveillance, for timely initiation of second line treatment, as well as for identification of patients for inclusion into clinical trials on novel agents for first line treatment of metastatic CRC [178, 179]. According to recent reports, patients with stage III CRC could be sub-dived into low- and high-risk groups, where low-risk patients would benefit from

reducing the duration of adjuvant chemotherapy from 6 to 3 months, without significantly increasing the risk of recurrence [180, 181]. Alongside the well-established biomarker carcinoembryonic antigen (CEA), the BRAF V600E mutation is currently the only molecular biomarker will clinically relevant prognostic value. The BRAF V600E mutation occurs a subgroup of CRC patients and confers an increased risk of developing recurrent disease. Using KRAS mutations expressed in RNA as biomarker in combination with BRAF V600E status, to support a shorter 3-month duration of adjuvant chemotherapy in low-risk patients, could potentially minimize toxicity, reduce treatment costs, and enhance quality of life, in a relatively large proportion of the patients with stage III CRC. Stage III high risk patients, on the other hand, might benefit from stage IV treatment protocols, including an intensified recurrence surveillance.

KRAS mutations have long been considered as an early event in the carcinogenesis of CRC [182]. The KRAS mutations in DNA of primary CRC tissues have been detected at similar levels in difference stages of CRC [183, 184]. In our studies, however, we found significant variability in the level of mutated KRAS mRNA in primary CRC tissues and a high-level of mutated KRAS mRNA was observed more frequent in stage IV metastatic CRC in comparison to earlier stages III, II and I (31.6% vs. 16.7%, 19.4% and 11.8%, respectively; $p = 0.0036$). There are several possible explanations to this discrepancy. From a converse perspective, the proportion of stage IV disease was higher in patients with a high level of mutated KRAS mRNA in the primary tumour tissue than in patients with a low or no expression of mutated KRAS mRNA (34.3% vs. 18.8%, $p = 0.0091$). This could imply that expression of mutated KRAS promotes invasion and metastasis, specifically in left-sided CRC tumours. Thus, the impact on invasiveness and metastatic potential would be conferred through expression of the mutant KRAS alleles, rather than by the DNA defect itself. A recently published mouse model of CRC, has identified the expression of mutant KRAS as a driver of invasion and metastasis, indicating that the level of continued expression of mutant KRAS could play a role in the development of metastasis through up-regulation of TGF-β signalling [185]. On the other hand, it is possible that the expression of mutated KRAS mRNA is activated only secondary to the accumulation of genomic changes at later stages of CRC. However, this hypothesis appears unlikely, since a high level of mutated KRAS mRNA was detected in similar or even lower frequencies in metastatic disease in comparison with earlier stages (22.2% versus 28.5%) among CRC patients in the same cohort but with right-sided tumours. The observed correlation between expressed KRAS mutations in primary CRC tumour tissue and prognosis of the patient, warrants further evaluation of expressed KRAS mutations as a biomarker for the purpose of predicting response to therapeutic agents targeting the mutant KRAS protein, as well as for accurate selection of high-risk patients for clinical trials on novel drugs targeting KRAS-mutant cancers in general.

Sensitive detection of expressed BRAF mutations in thyroid cancer

The BRAF T1799A (V600E) is most common point mutation of the BRAF gene in papillary thyroid cancer. The mutation activates the MAPK pathway causing a loss of control of cellular proliferation and triggering malignant transformation in the thyroid gland [78, 186, 187]. BRAF mutations are increasingly being used as biomarkers, but the prognostic relevance of the BRAF V600E mutation remains controversial in papillary thyroid carcinoma [73, 188-190]. The presence

of the BRAF V600E mutation is valuable for identifying patients, who in a relapse or primary metastatic setting, could be eligible for targeted BRAF inhibitor therapy with currently available drugs, such as lenvatinib, vemurafenib or sorafenib. BRAF V600E mutations have not, however, been confirmed as an independent predictor of poor outcome in TC [72].

Currently, Sanger sequencing is the gold standard for point mutation detection, due to the possibility to analyse multiple mutations simultaneously. Limitations of this method include a relatively low sensitivity, with a lower detection limit for mutated alleles of about 7–20% [191]. Due to tumour tissue heterogeneity the frequency of mutated alleles can be highly variable and, as a result, a significant number of low-level mutations will remain undetected by Sanger sequencing.

In thyroid cancer tissue samples, we detected expressed BRAF V600E mRNA mutations in 56.3% (18/32) and corresponding DNA mutations in 40.6% (13/32) of the patients, which is roughly similar to the prevalence reported in previous studies [144, 192-195]. The mRNA-based mutation detection assay thus contributed to a 28% improvement in the sensitivity of detection. In comparison, Sanger sequencing failed to detect the BRAF V600E mutation in 5 out of 18 samples, which were positive with the ExBP-RT-based BRAF V600E mRNA assay in our study. This discrepancy could be explained by the superior technical sensitivity of the mRNA-based assay compared to direct sequencing, but importantly also by the higher copy number of BRAF V600E mRNA transcripts compared to the copy number of BRAF V600E DNA in thyroid cancer cells. The detection of the BRAF V600E mutations in mRNA without prior amplification has previously been demonstrated using a nanomechanical sensor comprising of microcantilever arrays [196]. This device enables sensitive detection of mRNA at a concentration of 20 ng/µl and recognition of mutated BRAF DNA in a 50-fold excess of the wildtype background.

No expression of KRAS or BRAF mutations in potential in pre-malignant conditions of the esophagus

Despite the emerging role of KRAS and BRAF mutation in biology and cancer research, most studies reported a less important role of KRAS and BRAF mutations in the development of some upper digestive tract cancers, such as esophageal squamous cell carcinomas (ESCCs) and basaloid squamous cell carcinomas (BSCCs), including [197-200]. In esophageal adenocarcinoma (EAC) and associated high grade intraepithelial lesions, KRAS codon 12 mutations occur at a frequency between 30 and 40% and might represent a late event in the Barrett's metaplasia-dysplasia-adenocarcinoma sequence [61]. Due to the limitation of current detection techniques and the amount of research data, the role of these mutations in pre-malignant conditions is still unclear. Therefore, we decided to utilize the ExBP-RT technique using mRNA as material for detection mutations at KRAS codons 12 and 13, as well as BRAF V600 in adult patients treated for esophageal atresia in infancy. The hope was that it would be useful for identifying patients at risk of developing malignant transformation. Despite the high sensitivity of ExBP-RT assay, we could not detect tissue RNA expression of KRAS or BRAF mutations in any of the 112 endoscopic biopsies from 61 young adults that had been treated for EA in infancy. This is the first study describing the mutational status of KRAS and BRAF in patients treated for EA. While histological analysis revealed gastric metaplasia in 10/61 cases and intestinal metaplasia in further 5/61 cases,

neither dysplasia nor EAC was found in any of these patients. At the time of sampling, the mean age of this cohort of patients was 35.6 years and the negative results reported here might reflect the fact that no KRAS-related malignant transformation had taken place in any of the 61 patients included in the study. In a previous study by Trautmann et al., KRAS codon 12 mutations were only found in 1/252 (0.4%) metaplasia patients and 4/105 (3.8%) dysplasia patients [201]. In addition, Aber et al. detected KRAS mutations in only 1/39 (2%) patients with Barrett's esophagus [198]. Our results are consistent with these findings, emphasizing that the KRAS and BRAF genes alone, might be rare or absent in pre-neoplastic conditions of the esophagus and might not be ideal markers for malignant transformation in patients with EA. It is likely that more studies will be needed in order to reveal genetic markers or combinations of markers that can reveal early signs of malignant transformation, in order to identify patients in need of early intervention.

CONCLUSIONS

We have demonstrated that the level of mutated KRAS mRNA in the primary tumour tissue strongly correlates with prognosis in left-sided CRC. In contrast to DNA-based KRAS mutation testing, this novel biomarker can stratify patients with left-sided stage III CRC into distinct risk groups, thus creating a possibility for adjuvant treatment planning based on individualized risk assessment. Moreover, it could provide a useful tool for selection of high-risk patients for clinical trials on novel targeted therapies for KRAS-mutant cancers. These findings highlight the potential of expressed mutations as a novel class of biomarkers. We have also successfully established a novel assay for ultrasensitive detection and quantification of the BRAF V600E mRNA in FFPE tissue from thyroid cancer. This assay not only reveals the presence of the BRAF V600E mutation, but also the level of the mutated BRAF V600E mRNA. This approach opens new possibilities to study the functional consequences of mRNA expression of mutated genes and the potential clinical utility of mutation detection in mRNA with an increased sensitivity as compared to DNA-based approaches. When analysing endoscopic biopsy samples taken from young adults under follow-up after surgical treatment for esophageal atresia, which represent conditions with an increased risk of malignant transformation, we could detect neither expressed KRAS nor BRAF mutations at a reliable level in the patient samples. The absence of expressed KRAS and BRAF mutations gives no indication of malignant transformation occurring in this patient cohorts, but further follow-up will reveal whether any of the patients will eventually go on to develop cancer.

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